BIOCHEMICAL AND HISTOLOGICAL INVESTIGATIONS OF VIRAL LOCALISATION IN THE HYPERSENSITIVE REACTION OF *PHASEOLUS VULGARIS L. VAR PINTO* TO TOBACCO MOSAIC VIRUS INFECTION.

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ABSTRACT

STOBBS, Lorne,W *Biochemical and Histological Investigations of viral localisation in the hypersensitive reaction of Phaseolus vulgaris L. var Pinto to tobacco mosaic virus infection.* The infection of *Phaseolus vulgaris L. var Pinto* with tobacco mosaic virus(TMV) results in the production of distinct necrotic lesions confining the virus to restricted areas of the leaf surface. Biochemical and histological changes in the leaf tissue as a result of infection have been described. Trace accumulations of fluorescent metabolites, detected prior to lesion expression represent metabolites produced by the cell in response to virus infection. These substances, are considered to undergo oxidation and in diffusing into adjacent cells, react with cellular constituents causing the death of these cells. Such cellular necrosis in advance of infection effectively limits virus spread. Chromatographic studies on extracts from TMV infected Pinto bean leaf tissue suggests that a number of extra-fluorescent metabolites produced on lesion expression represent end products of phenolic oxidation reactions occurring earlier in these cells. Inhibition of phenolic oxidation by ascorbate infiltration or elevated temperature treatment resulted in the absence of extra-fluorescent metabolites and the continued movement of virus in the absence of necrosis. Further studies with

ascorbate infiltration indicated that irreversible necrotic events were determined as early as 12 to 18 hrs after viral inoculation. Histochemical tests indicated that callose formation was initiated at this time, and occurred in response to necrotisation. Inhibition of necrosis by either ascorbate infiltration or elevated temperature treatment resulted in the absence of callose deposition.

Scanning electron micrographs of infected tissue revealed severe epidermal and palisade cell damage. Histochemical tests indicated extensive callose formation in cells bordering the lesion, and suggested the role of callose in the blockage of intercellular connections limiting virus movement. The significance of these cellular changes is discussed.

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INTRODUCTION

> *The symptoms of disease are marked by purpose, and the purpose is beneficent. The processes of disease aim not at the destruction of life, but at the saving of it.*
>
> *Frederick Treves(1905)*

Whenever a plant virus interacts with a susceptible host, there are two possibilities of symptom development. Virus may spread over the entire plant evoking systemic symptoms which may result in the death of the plant, or it may produce necrotic lesions at its point of entry resulting in the restriction of the infecting virus to these lesions. The development of local necrotic lesions is little understood, and is regarded as a protective mechanism localising the infective agent to restricted areas of the host tissue sensitive to the pathogen. This latter response, reflecting the so called *hypersensitive reaction* is believed to be one of the most important defence mechanisms of the plant to infection(Farkas,Kiraly, and Solymosy,1960). Müller (1959) defines hypersensitivity as all morphological and histological changes that, when produced by an infectious agent, elicit the premature dying of the infected tissue as well as the inactivation and localisation of the infectious agent.

The formation of local lesions has been described as one of the most striking symptoms of virus diseases(Solymosy,Farkas, and Kiraly,1959). Despite numerous biochemical and histological studies, the specific changes in the host tissue in confining

virus spread remain obscure. The questions arise *What are the processes leading to this important defence mechanism in plant tissues? What is the nature of these processes, at what time do they occur during the infection process, and what is their association with lesion formation?* It is with these questions in mind that the following study has been undertaken in the hope that the role of the necrotic reaction on the mechanism of viral hypersensitivity in plants would be more clearly understood.

REVIEW OF LITERATURE

The importance of studying the physiology of plant disease resistance is well documented in the literature (Solymosy,1960; Israel and Ross,1967; Loebenstein,1972). Only recently, however has attention been focused on the elucidation of the cytochemical and structural changes occurring in virus infected hypersensitive host plant tissue. Such changes, leading to the development of necrotic lesions and localisation of virus in infected plant tissue, remain poorly understood. The association of necrotic lesions with viral localisation has led to the development of the theory of hypersensitive localisation. Hypersensitivity to viral parasites has been characterised as a host protective mechanism involving rapid necrosis of one or more cells of the host at the penetration site accompanied by the restriction of viral movement. For sake of convenience, hypersensitive interaction has been reviewed under two separate titles (a) biochemical and (b)histological.

(a) Biochemical studies

Biochemical studies of the hypersensitive reaction are almost unanimous in emphasising the role of phenol metabolism in plants (Solymosy,Farkas,and Kiraly,1959; Solymosy,1960; Farkas, Kiraly and Solymosy,1960; Parish,Zaitlin and Siegel,1965; Simons

and Ross,1970; Frigit,Legrand and Hirth,1972; Frigit,Gosse, Legrand and Hirth,1973). Increased synthesis of aromatic compounds such as polyphenols, flavonoids, etc.,coincident with the stimulation of enzyme oxidizing polyphenols in viral infected tissue, are probably the most widespread responses of hypersensitive host tissue. Studies in hypersensitive hosts stress the possibility that oxidation-reduction systems acting on polyphenols are altered in the diseased tissue, resulting in the accumulation of toxic polyphenolic oxidation products killing the host cells(Solymosy et al.,1959; Solymosy,1960; Farkas et al.,1960; Parish et al.,1965; Simons and Ross,1971; Frigit et al.,1972; Spurr and Main,1974). The induction of tissue necrosis in a ring of non-infected cells located perimetric to the infection site is probably responsible for the cessation of lesion expansion and limitation of viral spread into adjacent tissue(Farkas et al.,1960).

Evidence for or against the *in vivo* oxidation of phenolic compounds is rarely conclusive. One of the major obstacles in making an accurate assessment of the *in vivo* oxidation of phenolic compounds and their possible oxidation products during pathogenesis is that the product of such a reaction has not been unequivocally demonstrated. Apart from research on a few individual compounds(Best,1944,1963; Gill,1965; Hampton,1970; Tanguy and Martin,1972) no systematic identification of viral

hypersensitive products can be found in the literature. Such an inadequacy may relate to the possible complexity of the phenolic oxidation products.

Accumulations of scopoletin, a normal constituent of many plants, has been reported around the primary lesions of tomato spotted wilt virus(TSWV) and tobacco mosaic virus(TMV) lesions on *Nicotiana glutinosa*(Best,1944,1963). Hampton,Suseno and Brumagen(1964) noted accumulations of two unidentifiable fluorescent compounds in TMV infected hypersensitive tobacco plants. Both compounds appeared at the time of lesion formation and were localised around the necrotic lesions. Neither compound was found to react with phenolic reagent indicators.

A comprehensive account of the fluorescent metabolites in virus or rust infected bean leaves was presented by Gill(1965). Studies on French beans infected with tobacco mosaic virus(TMV), bean strain of tobacco mosaic virus(BTMV), and bean rust *(Uromyces phaseolus Pers Wint.)* indicated a definite range of extra-fluorescent metabolites associated only with the virus hypersensitive reactions. The most prominent metabolite was occasionally present in trace amounts after light mechanical injury of the leaf surface. A number of metabolites were found to be pathogen non-specific. The identification and relationship of these metabolites to the hypersensitive reaction has not been demonstrated.

Hampton(1970) produced evidence for the *in vivo* oxidation of chlorogenic acid to a protein-quinone complex in tobacco plants infected with tobacco streak virus(TSV). Symptomless, systemically infected leaves contained relatively smaller amounts of this product than did leaves exhibiting severe necrosis. The product was not detected in healthy leaves. Hampton(1970) concluded that the oxidation of phenolic compounds is either initiated or accelerated in tobacco by infection with TSV.

Complexes formed between oxidized phenols, proteins, and possibly other constituents resulted in brown pigment formation and necrosis in tobacco cultivars infected with the fungal pathogen *Alternaria alternata*(Spurr and Main,1974). Such pigment formation is believed to be basic to lesion development although pigment induction may be a non-specific plant process activated by many types of infections or injuries.

The hypersensitive reaction of soybean leaves to *Pseudomonas glycinea*, as studied by Keen and Kennedy(1974), was typified by rapid accumulation of several isoflavonoid compounds as hydroxyphaseollin, coumestrol, daidzein, and sojagol. The authors felt that these phytoalexin compounds are causally related to the restriction of bacterial populations in hypersensitively responding soybean leaves.

Possible aberrations of various phenolic biosynthestic

pathways induced by pathogen infection have been investigated by Brown and Tenniswood(1974). The authors, in analyzing free and bound phenolic acids and coumarins associated with crown gall tumour tissue of tobacco(*Nicotiana tabaccum*), suggest that coumarin biosynthesis might be diverted in favour of elevated levels of esculetin and scopoletin.

Although increases in phenolic compounds at the infection site have been known for some time, their involvement in the defence mechanism of plants to disease has not been demonstrated. The induction of various phenolic changes in advance of infection has been amply established(Solymosy and Farkas,1963;Israel and Ross,1967; Ross and Israel,1970; Spencer and Kimmins,1971). From these studies it can be inferred that the capacity for biosynthesis of phenols is increased in living cells bordering lesions at a time coinciding with the induction mechanism that eventually operates to limit lesion size. A great disparity is found in the literature regarding the time of induction of such a localising mechanism. Equally diversified are the experimental approaches to the problem.

Oparin(1921) demonstrated that various phenolic substances, while undergoing oxidation, were able to undergo reverse reduction at the expense of the hydrogen of the substrate. Later, it was speculated that if the hypersensitive reaction was a product of the activity of oxidizing enzymes, it should in turn be

inhibited by reducing compounds either generated endogenously in the host or by their exogenous application(Farkas et al., 1960;Parish et al.,1965). This inhibition of the hypersensitive reaction is apparently based on a chemical *in statu nascendi* reduction of the quinones formed in plants after infection(Solymosy et al.,1959). The major non-enzymatic constituent of plant tissues keeping phenolics in a less toxic reduced state is ascorbic acid. In fact the infiltration of ascorbate into virus infected hypersensitive foliar tissue greatly reduced lesion counts(Ryjkoff and Soukhov,1944;Farkas et al.,1960; Parish et al.,1965). These authors concluded that the polyphenol-polyphenoloxidase system is important in the mechanism of viral localisation.

An interesting discrepancy is seen in the literature on the ascorbate-lesion suppression effect. Parish et al(1965) noted a fifty percent inhibition of local lesion formation in ascorbate infiltrated *N. glutinosa* leaves inoculated with TMV. The lesions which formed were larger and less pigmented than lesions on water infiltrated controls. These observations differ from those reported by Farkas et al(1960), where ninety-five percent lesion reductions were observed. Parish et al (1965) interpret this difference to a lower concentration of ascorbate(5×10^{-3}M) used in their experiment, whereas Farkas et al(1960) used a more concentrated infiltrate(5×10^{-2}M).

However, such a difference could well be due to different experimental procedures. Farkas et al(1960) infiltrated *N. glutinosa* leaves prior to inoculation with TMV, whereas Parish et al(1965) infiltrated the leaves 24 hrs after inoculation. To my knowledge, no mention of this difference in experimental procedures is made in the literature.

Kosuge(1969) in referring to Parish's work, noted that lesion size should have been restricted by the enhanced reducing power supplied by the ascorbate; however this was not the case. Recognizing that Parish infiltrated the leaves 24 hrs after inoculation, a feasible explanation can be forwarded. According to the hypothesis of Farkas et al (1960), an accumulation of quinones associated with viral localisation would occur shortly after infection. Since ascorbic acid reduced quinones to a non-toxic form, the quinones formed in *N. glutinosa* at 24 hrs would be reduced by the exogenously applied ascorbic acid when the leaves were infiltrated 24 hrs after inoculation. This would destroy the quinone accumulations around the sites of virus penetration and localisation in the leaf. The virus, thus could spread outward, and on necrosis would result in a larger lesion.

The polyphenol-polyphenoloxidase hypothesis has been criticised by several researchers in light of various ascorbate studies. Milo and Santilli(1967) noted that TMV inoculated *Phaseolus vulgaris* L. var Pinto leaves exhibited increasing

ascorbate concentrations after inoculation, which subsequently decreased rapidly due to oxidation of ascorbate to dehydro-ascorbate. The authors rejected the role of ascorbate in the restriction of infection by comparing the time at which the virus becomes localised relative to the period of rapid oxidative change, as reflected by the ascorbic acid decrease. Sukhov and Kapitsa(1955) reported that all lesion forming events of TMV on *N. glutinosa* at 28°C. were concluded 8 to 10 hrs. after inoculation. Assuming by analogy that the period for the conclusion of lesion forming events in bean was equally short, Milo and Santilli assert that the period of rapid ascorbate oxidation takes place long after the virus has become localised, and long after the area which is to become a lesion has been delimited. It must,however,be pointed out that their measurements were made on half leaf samples and represent concentrations in the tissue as a whole. Such a sampling procedure would not have detected localised changes in ascorbate in the immediate regions of developing local lesions (Bawden,1964).

Treatment of virus infected leaf tissue with ascorbate solutions was found to have no inhibitory effect on polyphenol-oxidase activity and exhibited little effect on the synthesis of virus in the infection centres(Farkas et al.,1960). It becomes apparent that the process of viral multiplication and the development of local lesions may not be related since viral multiplication ocurred in ascorbate treated leaves without the formation

of local lesions(Milo and Santilli,1965). White(1968) in a review paper, asserts that a point of great significance is that no systemic movement of virus occurs from the infection centre where the necrosis is suppressed, implying that the marked polyphenoloxidase activity and consequent necrogenesis in local lesions is not responsible for confining the virus to the local lesion. It is difficult to decide on reading the paper, whether White refers the evidence supporting this statement to Farkas or to himself. Since Farkas did not investigate localisation of virus when necrotisation was suppressed, it can be assumed that the evidence is thus ascribed to White. However, no data is presented in White's paper and the significance of his statement is difficult to access.

Increases in phenolic activity in many diseased tissues has been attributed, at least in part, to enzyme protein synthesis triggered by the parasitic attack of the host. The relationship of such increases to necrotic lesion development was demonstrated by Shaw(1963) and Stahmann(1965). Formation of TMV lesions on the lower leaves of hypersensitive tobacco was followed by the development of resistance on systemically uninoculated leaves,accompanied by permanently increased peroxidase and catalase activity(Ross and Bozarth,1960;Bozarth and Ross,1964; Simons and Ross,1971). Induction of systemic resistance in the uninoculated leaves after inoculation of the lower leaves with TMV, was not, however accompanied by changes

in total phenols. Such peroxidase increases in the absence of change of phenolic levels is suggestive of spatial or functional separation of enzymes and their substrates(Loomis, 1966,1973; Pierpoint,1970;Keen and Kennedy,1974). Challenge inoculation of the upper resistant leaves however, affected an earlier and greater decrease in the concentration of phenolic compounds during and subsequent to lesion formation than that occurring when non-resistant leaves were similarily inoculated(Simons and Ross,1971). The authors suggest that this increased capacity for oxidation of phenols in resistant tissue constitutes a potential for a rapid hypersensitive response that is not expressed until virus induced cellular disruption brings the enzyme into contact with the substrate. Similar decompartmentalization models have been proposed for other hosts(Giazinazzi,Valle,and Martin,1969;Goodman,1968,1972; Keen and Kennedy,1974).

Studies indicate that polyphenoloxidase increases in the whole leaf after viral infection, these increases neither being restricted to the cells where viral multiplication occurs nor to the necrotic tissue forming the lesion(Van Kammen and Brouwer, 1964). Such increases although measureable only two to three days after inoculation are believed to be induced as early as 7 hrs. after inoculation(Van Kammen and Brouwer,1964). A slight increase in respiration due to lesion expression might indicate the involvement of such phenolases(John and Weintraub,

1967; Chant,1967). Several types of enzymatic activity have been reported in virus-host combinations: *a)* Increases in oxidase concentration(Van Kammen and Brouwer,1964; Novacky and Hampton,1968; Cabanne,Scalla,and Martin,1971; Spurr and Main,1974), *b)* changes in the relative amounts of different enzymes(Bates and Chant,1970), *c)* appearance of new peroxidases (Farkas and Stahmann,1966; Bates and Chant,1970) or new phenolases(John and Weintraub,1967). Highest peroxidase activity was recorded in hosts which responded with necrosis to virus infection(Bates and Chant,1970; Van Loon and Greelen, 1971) and was noted to increase with symptom severity(Yarwood, 1960; Loebenstein and Linsey,1966; Simons and Ross,1971). Chlorosis or cell death has been suggested as a trigger mechanism in the induction of certain peroxidases.

The basis for the induction of acquired resistance in viral infected tissue has received much attention. Such resistance has been attributed to possible by-products of viral synthesis(fragments of protein complements of the virus) that diffuse in advance of virus infection into the adjacent tissue(Hofferek and Proll,1967). However the lack of resistance specificity for different viruses provides strong evidence to the contrary(Ross,1961; Solymosy,1970). Synthesis of a substance(s) following initial virus infection has been suggested to be responsible for hypersensitive localisation. Later, when produced in larger quantities, it/they would diffuse from the primary infection site and induce resistance in adjacent tissue.

This substance may diffuse into the uninfected tissue and activate the localising mechanism in advance of virus spread in the cells(Loebenstein,1972). Activation of resistance by various non-multiplying substances which do not induce necrosis is in most cases limited to the tissue directly treated with the inducer. Resistance invoking viruses however activate resistance in non-invaded tissue possibly by a de-repression process of the host genome for localisation or resistance, normally in a repressed state(Solymosy,1970; Loebenstein,1972). Certain analogies have been suggested between the resistance induced by viruses or non-viral agents in plants and the interferon system in animals(Ross,1961; Sela and Applebaum,1962; Sela,Harpaz,and Birk,1965,1966; Kimmins,1969). However such relationships are difficult to resolve until the mode of action of a chemically defined agent has been established.

The inhibition of hypersensitivity at elevated temperatures in TMV inoculated *Nicotiana tabacum var Xanthi* plants has been studied in attempts to access the role of phenolic compounds and their associated oxidases to the necrotic process(Martin,1958; Cabanne, Scalla,and Martin,1971; Shimomura,1971: Tanguy and Martin,1972). Xanthi plants kept at 30 °C. and inoculated with TMV developed systemic infection with declining phenol levels. On transfer to 20 °C. the hypersensitive reaction was rapidly initiated, no accumulation of phenols was observed prior to visible necrosis, and oxidase levels remained unchanged. Phenols,

reached significant levels only after the appearance of necrosis(Cabanne et al,1971; Tanguy and Martin,1972). These authors conclude that changes in enzyme activity found during the hypersensitive reaction are a consequence not a cause of the death of the cell. Tanguy and Martin(1972) further assert that the formation of toxic quinones and their polymerised products are unlikely to be responsible for lesion development.

Systemic spread of virus from lesions exposed to elevated temperatures suggests that localisation of infection does not depend primarily on the death of infected cells(Shimomura,1971). Evidence seemingly suggests that localisation of TMV in its lesion hosts depends primarily on some virus limiting factor that acts in advance of cell necrosis. The nature of this limiting factor is not understood. The fact that local lesions developed on leaves previously systemically infected by incubation at elevated temperatures and then exposed to lower temperatures, is suggestive that a certain level of virus multiplication is a requisite to lesion formation(Shimomura,1971).

Knowledge regarding viral localisation is still fragmentary, and many uncertainties continue to exist in the interpretations of their mechanisms. The role of many phenolic compounds and their related oxidases described in this report have not unequivocally been established as disease resistance factors. Moreover, little attention has been given to the possible functions of phenolic compounds in host-parasite interactions. Proof is

needed to establish the identity of many of these phenolic compounds alleged to be involved in the defence responses to infection. New techniques may open possibilities for further study of these substances.

(b) Histological Studies

From numerous studies on TMV infection in local lesion hosts it can be inferred that virus infection induces various physiological changes within or around local lesions. Increasing attention however is being focused on cytological and ultrastructural alterations due to virus infection in an attempt to explain the blocking mechanism which effectively prevents virus from spreading beyond necrotic lesions. Localisation has been explained in terms of a reduction in the number of pathways available for viral spread(Spencer and Kimmins,1971). Such a process may be physical rather than physiological. Esau(1967) proposed that mechanical interruption of celluiar continuity would prevent virus spread from the infection centre. Virus spread, presumably via plasmodesmata would be restricted by the severing of the plasmodesmata by a wound healing process such as callose deposition(Currier, 1957). Support for this hypothesis was presented by Wu,Blakely, and Dimitman(1969) and Wu and Dimitman(1970), who gave evidence that the restriction of virus spread in Pinto bean primary leaves could be attributed to the rapid death of infected cells and the

rapid response of adjacent non-necrotic cells by initiating cell wall callose deposition. This callose would seal off the plasmodesmata between infected and non-infected cells. The association of callose substances with the hypersensitive response has been demonstrated by fluorescent microscopy(Wu et al,1969; Wu and Dimitman,1970; Hiruki and Tu,1972), and by electron microscopy(Spencer and Kimmins,1969; Hiruki and Tu, 1972). Spencer and Kimmins(1971) report that while they always observed callose in the regions of the sieve tube and at the base of leaf hairs, they only occasionally found callose plugs over plasmodesmata located in the resistant zone. From ultra-structural studies of TMV infected Pinto bean leaves, they suggest that the significant structural changes occurring in the resistant zone are those associated with paramural body formation. Subsequent severence of intercellular connections in the zone of cells bordering the infected cells would result from plasmalemma retraction and possible sealing off of the plasmo-desmata by paramural body accumulations.

Tu and Hiruki(1971) defined two types of cell wall thickening in potato virus M infected Kidney bean. Initial callose deposition on the inner wall of non-necrotic cells bordering semi-necrotic cells was described as a temporary seal formed in quick reponse to injury, later resulting in secondary wall thickenings which seal off the plasmodesmata. The second type, also reported by Esau(1967) was initiated by the appearance of boundary

formation on the secondary cell wall of semi-necrotic cells and was characterised by the roughening of the plasmalemma and accumulation of membrane bound vesicles and tubules. Complete plugging and severing of intercellular cytoplasmic connections would occur rapidly. Cytoplasm containing virus in such cells would thus be enclosed in thickened cell walls and effectively isolated(Tu and Hiruki,1971). It is suggested that these changes in cell wall structure are related to a series of events of localisation, initially by plasmodesmatal blockage by callose followed by more extensive secondary wall thickening involving boundary formation(Hiruki and Tu,1972).

The isolation of two protein-polysaccharide complexes from TMV infected Pinto bean leaves, and their role in hypersensitive localisation, was examined by Brown and Kimmins (1973). The induction of such glycoproteins has been shown to be independent of the presence of virus and is considered a product of the wounding process. The authors ascertain that if cell wall modifications are involved in localisation, through the reduction of available pathways for virus spread, such would depend on the interplay of two separate events, the rate of virus movement, and the time of cell wall modifications. If cell wall modifications occurred in advance of virus movement from the centre of infection, localisation may occur. However, if virus has spread in advance of the sites of new deposition, localisation would be incomplete. It was

inferred from these studies that localisation may occur only when virus transmission has also caused a wound response to the host(Spencer and Kimmins,1969; Kimmins and Casselman,1969). Such a process could explain why certain viruses are localised when mechanically inoculated on the host, but become systemic when introduced by grafting(Weintraub and Ragetli,1961).

Many uncertainties continue to exist in the interpretation of the mechanism of hypersensitive localisation. It is probable that tissue necrosis resulting from the hypersensitive reaction is linked in some way with the defence reaction of the host plant. Whether such necrosis of the tissue is a major factor in containing a virus in a limited area remains obscure. Whether modification of the cell wall causes localisation through the reduction in the number of available pathways for viral spread remains open to conjecture. Until further information becomes available, these suggestions must remain speculative.

In the preceding review, the discussion has been limited to what the author considers the most salient literature reports dealing with viral localisation, and concerned with those reports dealing with the metabolic and cytological changes induced by lesion forming events. Such collective accounts of the literature may offer a better understanding of the progress attained to date and may be useful in directing future advances in viral localisation research.

Quanjer(1913), in citing Goethe, appends:

> "I wish we would thoroughly appreciate the truth that we can never obtain complete comprehension unless we consider the normal and the abnormal at the same time and contrasted with each other."

MATERIALS AND METHODS

A. PLANT STOCK

Bean plants(*Phaseolus vulgaris L. var Pinto*)[1] were grown in vermiculite in flats and used approximately 10 days after germination. Plants were maintained in a growth chamber at 20°C. ± 2°C. and illuminated for a 16 hr. day with 800 ft. candles of cool-white fluorescent light, supplemented with 100 ft. candles of incandescent light. Plants were fed weekly with a nutrient solution(Appendix 2).

B. PATHOGEN CULTURES

Four strains of tobacco mosaic virus(TMV) were obtained from the Canada Department of Agriculture Research Station, Vineland Station, Ontario. These included TMV apple strain, MacNeill strain, tomato fruit lesion strain, and Holmes strain. Preliminary tests showed that tomato fruit lesion strain was the most infectious of the four strains on Pinto bean leaves and it was subsequently used for the tests. Virus inoculum was maintained on *Nicotiana tabacum cv. harrow velvet* under greenhouse conditions in isolation. Only the apical leaves were used for TMV inoculum preparations.

Cultures of bean rust(*Uromyces phaseoli*) and blight(*Xanthomonas phaseoli*) used in preliminary investigations on the phenolic content of infected plants were kindly supplied by Dr. C.E.Yarwood

1...for seed source, see appendix 1

Department of Plant Pathology, University of California, and Dr. M. Sutton, National Research Council of Canada, Ottawa.

C. GENERAL PROCEDURES

1. Inoculation

Inoculum was prepared from apical leaves of TMV infected *Nicotiana tabacum* plants showing chlorotic mottle symptoms. Infected leaves were macerated in 1% aq. K_2HPO_4 solution supplemented with 5%(v/w) Celite 545(Fisher Scientific C-212). Bean leaves predarkened for 24 hrs. prior to TMV inoculation were used as test plants. A one inch soft bristle brush(Canadian Tire Corp.#9-0331) saturated with the inoculum was used to apply an even layer of the inoculum across the primary leaf surfaces with uniform pressure. Control leaves were inoculated with 5% celite solution suspended in potassium phosphate solution. The leaves were rinsed in running tap water after inoculation.

2. Vacuum infiltration of ascorbic acid into bean leaf tissue.

Pinto bean leaves were grown in vermiculite in flats until the primary leaves were well developed. Plants were then removed from the vermiculite and vacuum infiltrated by inverting the plant and immersing the leaves in the infiltration solution. The roots were kept moist. A vacuum of -8mm Hg. was

maintained for 5 min. to ensure complete infiltration. Following infiltration the plants were placed in nutrient solution supplemented with the infiltration agent.

3. Chromatographic Analysis of Phenolic Metabolites

i. Extraction Procedures

Primary bean leaves were inoculated with TMV to produce between 600 to 1000 lesions per leaf. Ethanol extracts were prepared from a modification of the method used by Linskens(1959) as reviewed by Gill(1965). Ethanol extracts were not taken to dryness as suggested but adjusted to approximately 90% aq. ethanol and stored at 0 °C. until required.

ii. Hydrolysis of the extracts

Alkaline hydrolysis was performed at room temperature with 2N NaOH for 3 hrs. under a nitrogen atmosphere. The sample was then acidified with 2N HCl(pH 2), and the aglycone fraction extracted with ethyl acetate; the ethyl acetate washings being taken to dryness under reduced pressure and the residue taken up in 95% ethanol and stored at 0 °C.(Gill,1965).

Acid hydrolysis was achieved by refluxing for 2 hrs. with 2N H_2SO_4 followed by ethyl acetate extraction of the aglycone. The extracts were evaporated to dryness under reduced pressure and the residue dissolved in 95% ethanol and stored at 0 °C.(Gill,1965).

iii. Chromatographic separation of phenolic compounds

The phenolic acids and coumarins were separated by the use of two-dimensional descending paper chromatography. One hundred and twenty microlitres(120ul)of the test solution was spotted on the starting point of 46 x 57 cm. Whatman No.1 chromatography paper and developed in the first and second directions in 1-butanol:acetic acid:water(100:27:73) (BAW) and in 2% aq. acetic acid respectively (Gill,1965). One hour equilibriation was allowed for each solvent. Chlorogenic acid(Sigma Chemicals C-3878) was used as a standard.

iv. Identification of the Phenolic Compounds

The dried chromatograms were examined in long wave ultraviolet light(Blak Light Inc. B-100A) before and after fuming with ammonia vapor. Duplicate chromatograms were sprayed with one of the following reagents[2]: Arnow's reagent, ninhydrin, and the diazonium salts *p*-nitroaniline and sulfanilic acid both oversprayed with 20% Na_2CO_3. Spots were defined on the chromatography sheets, and their R_f mobility, UV fluorescence and spray reagent colour reactions recorded.

v. Analysis of the Extra-Fluorescent Metabolites

a. Isolation

2...For reagent preparation, see Appendix 3.

Gill(1965) reported the increased solubility in di-ethyl ether of the extra-fluorescent metabolites resulting from tobacco necrosis virus(TNV)infected bean leaves. This property coupled with increased mobility noted in 15% acetic acid allowed for the further purification of these spots. Using a similar procedure, the extra-fluorescent metabolites were extracted from unhydrolised ethanol extracts by several washings of di-ethyl ether. The combined ether extracts were taken to dryness under vacuum, leaving a yellow-brown residue with a sweet aromatic odor. The residue was redissolved in 95% ethanol and 120ul spotted on chromatography sheets. Development in the first direction was in BAW(100:27:73) with irrigation in the second direction for 17 hrs. in 15% acetic acid to maximize separation.

Spots were located on the paper under UV light and the desired compounds eluted in 70% ethanol. The eluate was taken to dryness under vacuum and the residue adjusted to 90% ethanol. Samples were stored at 0°C. prior to chromatography.

b. Characterisation

Samples of the several isolated compounds were chromatographed in one dimension on Whatman No.1 paper in the following solvents: BAW(100:27:73), 15% acetic acid, and six chromatography solvents used in phenolic investigations by Reio(1958):

Solvent F ethyl methyl ketone:diethylamine(921:79)

Solvent A methyl isobutyl ketone:4%formic acid (1000:100)
organic phase used as mobile phase

Solvent B chloroform(+1% ethanol):methanol:water: formic acid (1000:100:96:4)
organic phase used as mobile phase

Solvent C benzene:ethyl methyl ketone:2% formic acid (900:100:100)
organic phase used as mobile phase

Solvent D benzene:2% formic acid(1000:100)
organic phase used as mobile phase

All systems were descending and one hour equilibriation with the water phase of the solvent was allowed before addition of the mobile phase. Following UV examination, duplicate chromatograms were sprayed with one of the following reagents: Folin Ciocalteu reagent, 2,4-dinitrophenolhydrazine, sodium molybdate, ammonium molybdate reagent, Arnow's reagent, Ehrlich's reagent, phosphomolybdate, 1% potassium permanganate, 2% ferric chloride, 1% alcoholic aluminum chloride, or sprayed with diazotised sulfanilic acid or diazotised *p*-nitroaniline followed by an overspray with 20% Na_2CO_3 (Appendix 3).

4. Histological Examinations

Methods of growing Pinto bean and of inoculation of the primary leaves with TMV have been described. Inoculated plants used in the studies were maintained in a growth chamber at 22°C. ± 2°C. receiving continuous illumination of 800 ft.

candles fluorescent light supplemented with 200 ft. candles of incandescent light.

i. Tissue Preparation

Tissue samples($5mm^2$) incised from healthy, injured, and infected leaves were plunged into boiling 95% ethanol for 3 min. and decolourized in three washings of ethanol. The specimens were dehydrated in an ethanol series and cleared in terpineol. Tissues were embedded in paraffin blocks and sectionned with a Spencer 820 microtome. Sections 8u thick were found to be best suited for fluorescent and light microscopy. Sections were floated on a water bath at 45 °C. containing 1% Mayer's albumin and transferred to glass slides.

ii. Light Microscopy of Healthy and Infected Tissue

Sections used for light microscopy were stained in aniline blue(as described for callose stain) and used in conjunction with fluorescent microscopy observations. Light micrographs were taken with Kodak Panatomic X(ASA:32) with a Wild M-20 light microscope coupled with a Wild Dual Illuminator light source. A low voltage 20 watt light source was used.

iii. Fluorescent microscopy of surficial leaf tissue

Leaf samples bearing lesions at various stages of development were examined under UV illumination in a Wild M-20 light microscope. No preparative steps were taken to fix the tissue, leaf samples were placed directly on glass slides dorsal surface upwards.

iv. Microscopic Observations of Callose

a. Callose Stains

i. Light microscopy

Following de-waxing and hydration of the sections, staining for callose was accomplished with lacmoid(Gifford and Esau.1953) and resorcinol(Eschrich and Currier,1964) as outlined by the authors. Sections were wet mounted in an aqueous base mountant(Gurrs Water Mounting Media G-953, Baird and Tatlock) and observed under a Leitz orthoplan microscope.

ii.Fluorescent microscopy

A 0.01% solution of water soluble aniline blue(British Drug House,Toronto) made in M/15 K_2HPO_4 was adjusted to pH 9.5 with K_3PO_4. Hydrated sections were stained for 2 to 3 hrs. at room temperature. Sections were mounted in Uvak R11-45 aqueous fluorescent mountant(Edward Gun Ltd.,London) and observed under a Wild M-20 light microscope

fitted with a fluorescent assembly. A high pressure mercury vapor lamp(HBO-200w) served as the illuminator. Two excitor filters(UGI 2mm) with maximum transmissibility at 366nm, absorbed the visible spectrum. A barrier filter(OG1) was placed in the ocular tube of the microscope. Due to the gradual fading of callose fluorescence on exposure to ultraviolet radiation, micrographs were taken on Kodak Tri-X film(ASA-400) over a 2 min. exposure period.

v. Scanning Electron Microscopy

Leaf samples($5mm^2$) were fixed under mild vacuum at room temperature in 3% glutaraldehyde in 0.5M phosphate buffer(pH 6.8). Following washing for 10 minutes in several changes of buffer solution tissue samples were post-fixed in 1% OsO_4 in phospahte buffer for 1 hour.

In a preliminary investigation, sample tissues were prepared using four different procedures. The method of Lott and Darley, (1973) was adapted for bean leaf tissue. Tissue was plunged into isopentane cooled to -170 C. and rapidly transferred to a flask containing liquid nitrogen. The flask containing the specimen was placed under vacuum and upon solidification of the nitrogen, the vacuum was continued until the nitrogen had sublimated and the flask had returned to room temperature. The desiccated specimen was prepared for coating. Critical point drying was performed on two sets of samples dehydrated in either

an ethanol/CO_2 series or an acetone/freon series. A further sample was dehydrated in an acetone series and air dried.

Specimens were mounted on aluminum stubs and coated under vacuum on a rotary stage with a film of gold-palladium. Examinations were carried out on an AEI Scanning electron microscope(Model 1000) at 20-30Kv with a minimum beam current and the smallest aperature consistent with satisfactory image. The beam angle was varied continuously to determine structural conformation. Photographs were recorded on 75mm. black and white film.

Critical point drying using an ethanol/CO_2 series was found to give superior results to the other methods tested, and was used in the preparation of the experimental tissue.

vi. Electron microscopy..observation of virus particles in lesion tissue, sampling procedure:

Sample tissue(1cm^2) containing single developed lesions was embedded in paraffin as described previously. Fifteen micron sections were taken horizontally through the lower and upper epidermis, palisade, and mesophyll layers. Sections were mounted on glass slides and following de-waxing in Xylene, the sections were rehydrated. A drop of 2% phospho-tungstic acid(PTA,pH 7.0) was added to each slide and the sample tissue macerated with a glass rod. The suspension was applied to a carbon coated grid by the dip method(Hitchborn and Hills,1965).

RESULTS

A. CHROMATOGRAPHIC ANALYSIS OF PHENOLIC METABOLITES

1. Unhydrolized Extracts

A total of 13 different fluorescent metabolites were recorded in TMV infected bean leaves. Nine metabolites (designated 1-9,Fig.1) were recorded in healthy, celite inoculated, and TMV-celite inoculated leaves. Many of these metabolites have been identified with metabolites previously reported by Gill(1965)(2,4to9,Fig.2). Metabolites 2,4,5 were tentatively identified as quercetin derivatives and metabolites 6 and 7 as derivatives of caffeic acid and *p*-coumaric acid respectively. The fluorescent properties of metabolite 6 were typical of chlorogenic acid and the presence of an *o*-dihydroxy group was confirmed by the production of a rose-tan colour with Arnow's reagent. Co-chromatography with chlorogenic acid however indicated a difference in R_f mobilities in BAW(100:27:73) and 2% acetic acid. As suggested by Gill(1965) the evidence is strongly in favour of this unknown being a dipepside of caffeic acid. The failure of metabolites 1,3,7,and 9 to react with Arnow's reagent indicates the lack of an *o*-dihydroxy group.

Mild celite abrasion produced no gross quantitative changes in the pattern of the fluorescent metabolites. Four additional metabolites(designated 10 to 13,Fig.1) occurred in TMV infected bean leaves. Metabolite 10 was present intermittently in TMV-celite inoculated leaves, and was detectable only in trace amounts.

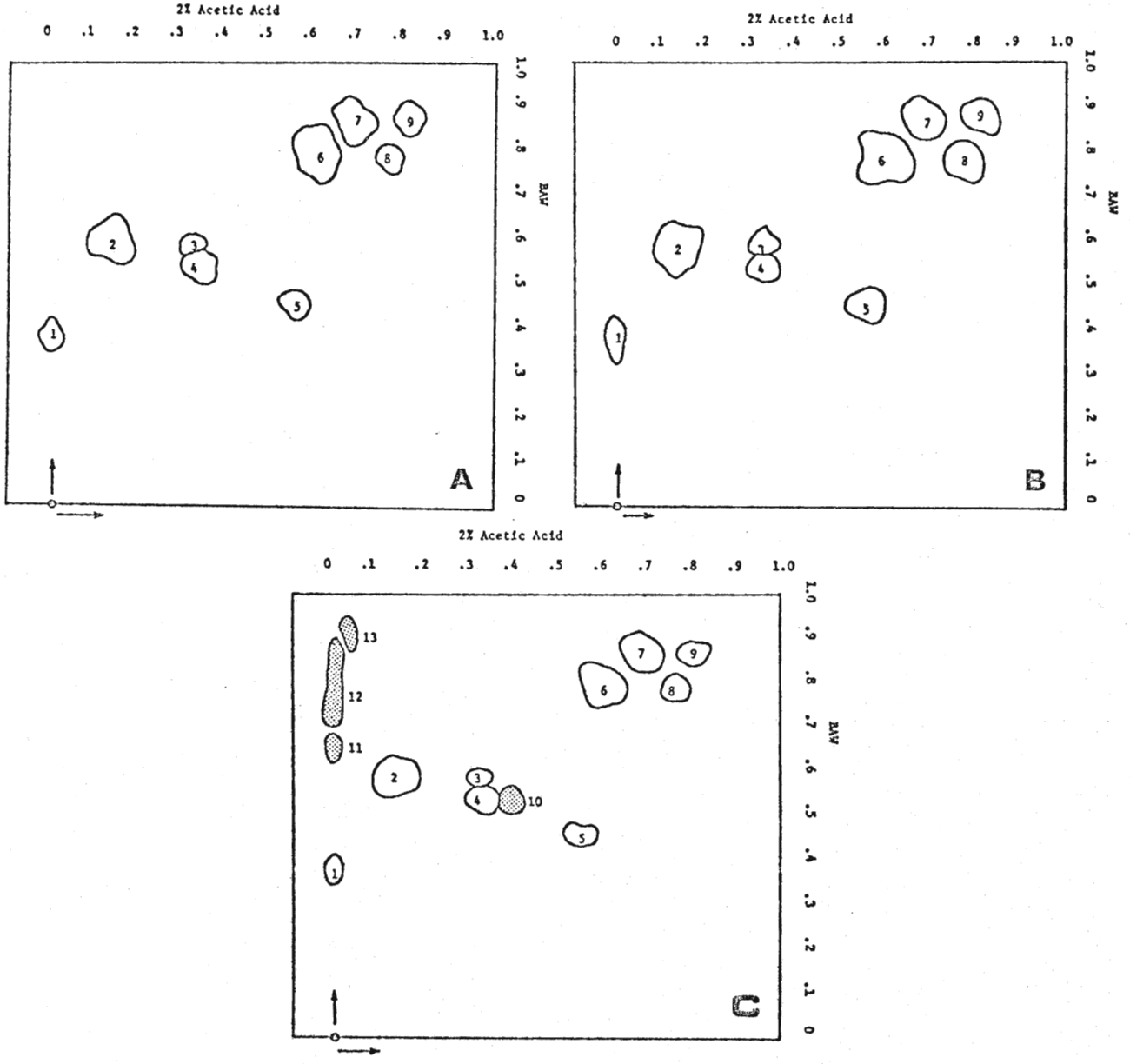


Fig.1 Diagrams of phenolic metabolites on two dimensional chromatograms from Pinto bean leaves. (A) Healthy, (B) Celite wounded, (C) TMV infected leaf extracts. Stipled spots are fluorescent metabolites recorded from TMV infected leaves only. Chromatograms were developed in 1-butanol:acetic acid:water, 100:27:73 (BAW) in the first direction, and in 2% acetic acid in the second direction.

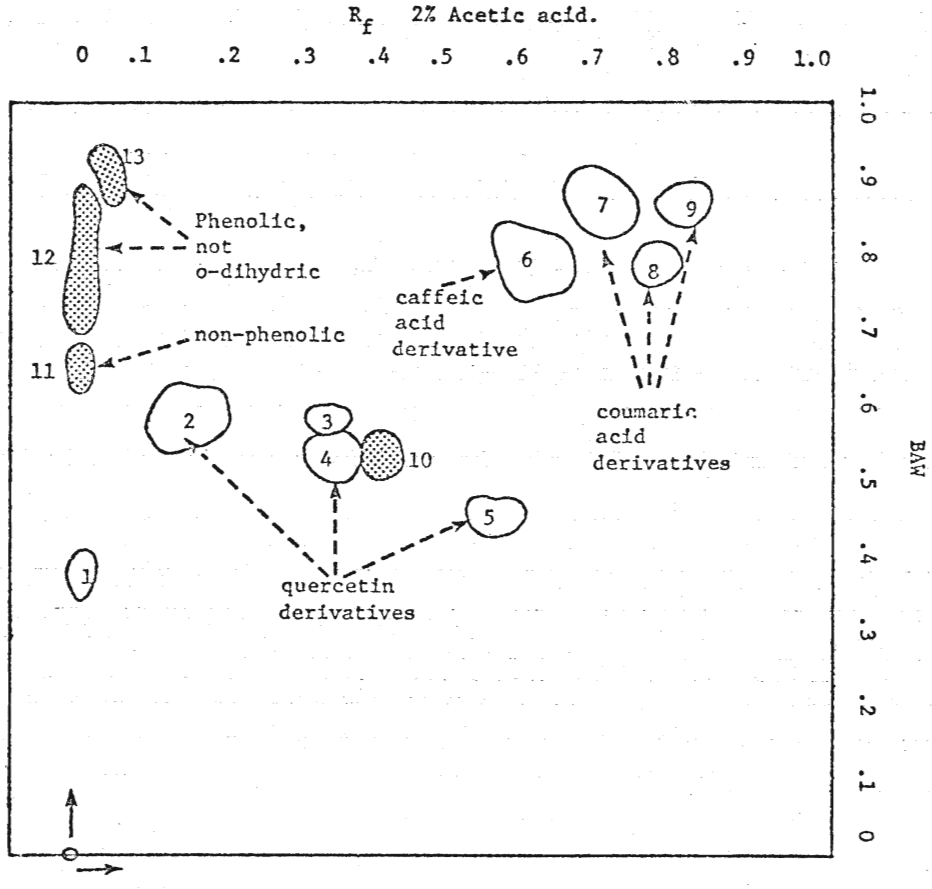


Fig.2 Characterisation of fluorescent chromatographic spots based on identification by Gill(1965). Stipled spots are fluorescent metabolites recorded from TMV infected leaves only. Chromatograms were developed in 1-butanol:acetic acid:water, 100:27:73 (BAW) in the first direction, and in 2% acetic acid in the second direction.

Tailing of these extra-fluorescent metabolites made their properties difficult to access. R_f mobilities proved most useful in their characterisation(Table 1a,b). R_f values represent the mean of a large number of determinations under the carefully controlled conditions described(See Materials and Methods). Visible and fluorescent colour reactions of substances on treatment with chromogenic sprays are shown in Table 1a,b. No reactions were apparent between the extra-fluorescent metabolites and the spray reagents.

Increased necrosis resulting from either severe abrasive damage or increased lesion density was associated with increases in certain metabolites(6,11-13,Fig.1) No quantitative determinations were made.

2. Acid Hydrolysis

On acid hydrolysis of the ethanolic extracts, 19 separate metabolites were characterised on paper chromatograms. A total of 17 spots(1-16,18,Fig.3) were found common to healthy, celite inoculated,and TMV-celite inoculated leaves. Two extra-fluorescent metabolites(17,19) present in TMV-celite inoculated leaves were masked by a pale, yellowish brown streak, non-fluorescent in ultraviolet light. Metabolite 17 was occasionally noted in trace amounts after mechanical injury. Increasing intensity of this compound on paper chromatograms was correlated with increased lesion density. R_f mobilities and spray reagent reactions of these spots are shown in Table 2a,b. Reaction of

Table 1a Characterisation of major fluorescent spots from healthy bean leaf extracts (T) trace, (F) faint, (P) pale, (L) light, (B) bright.

No.	R_f BAW	R_f 2% Ac	Colour	U.V.	U.V. plus NH_4OH (vp)	Nitro-aniline	N.A. plus 20% Na_2CO_3	Sulfanilic Acid	S.A. plus 20% Na_2CO_3	Arnow's Reagent	Ninhydrin
1	.40	0	-	F. yellow	yellow-rose	F. yellow	F. yellow	-	-	-	-
2	.60	.15	-	Brown	Brown-yellow	Yellow-tan	Rose-tan	Yellow-tan	Yellow-tan	F. Yellow-rose	-
3	.58	.32	-	F. pink	F. pink	-	-	-	-	-	-
4	.53	.35	-	Brown	Brown-yellow	F. tan	Rose-tan	T. rose to F. yellow	yellow-tan to rose	F. sepia	-
5	.48	.55	-	Brown	Brown-yellow	F. yellow-tan	Rose-tan	Yellow-tan to rose	Yellow-tan to rose	F. sepia	-
6	.80	.62	-	Blue-white	Olive-green	Yellow-tan	L. rose-tan	L. rose	L. rose	Rose-tan	-
7	.88	.70	-	-	P.Blue	F. purple	F. purple	F. tan	L. rose	-	-
8	.79	.79	-	F. blue-white	F. olive-green	F. olive	L. yellow	-	-	F. yellow-tan	-
9	.87	.83	-	-	-	F. purple	F. purple	F. tan	L. rose	-	-

Table 1b Characterisation of extra-fluorescent spots from TMV infected bean leaf extracts (F) faint, (P) pale, (L) light, (B) bright.

10	.55	.41	-	F. tan	P. green	-	-	-	-	-	-
11	.65	0	-	L. purple	L. purple	-	-	-	-	-	-
12	.80	0	-	B. purple	B. purple	-	-	-	-	-	-
13	.91	.25	-	Olive-green	Olive-green	-	-	-	-	-	-

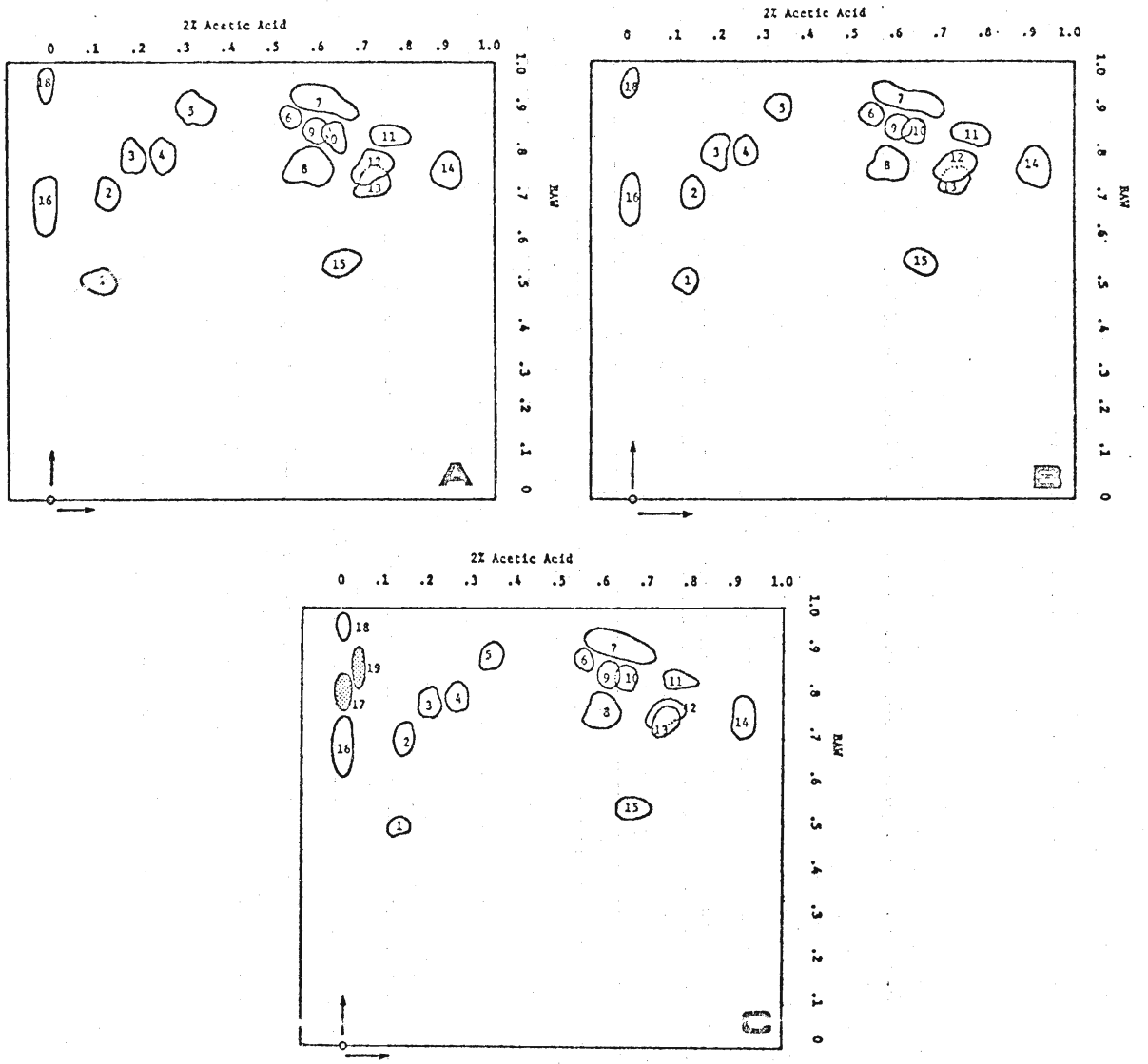


Fig. 3 Diagrams of fluorescent spots on two dimensional chromatograms from bean leaves. (A) Acid hydrolysis extract from healthy leaves, (B) Acid hydrolysis extract from celite wounded leaves, (C) Acid hydrolysis extract from TMV infected leaves. Stipled spots are fluorescent metabolites recorded from TMV infected leaves only. Chromatograms were developed in 1-butanol:acetic acid:water, 100:27:73 (BAW) in the first direction, and in 2% acetic acid in the second direction.

Table 2a Characterisation of major fluorescent spots from acid hydrolized healthy bean leaf extracts (VF) very faint, (F) faint, (L) light, (M) medium, (B) bright, (D) deep, (ID) indeterminate.

No.	R_f BAW	R_f 2% Ac	Colour	U.V.	Nitro-aniline	N.A. plus 20% Na_2CO_3	Sulfanilic Acid	S.A. plus 20% Na_2CO_3	Arnow's reagent	Hoeptner's reagent	2,4-dinitro phenyl hydrazine	Phospho-molybdate	Folin Ciocalteu reagent
1	.51	.12	-	F.purple-brown	L. tan	Tan	Yellow-tan	Tan	F. yellow	-	Yellow	-	-
2	.70	.13	-	F.blue-white	L. tan	Tan	L. tan	yellow-tan	F. sepia	-	-	-	F. yellow
3	.78	.19	-	F.white to L. purple	D. tan	L. rose	L. sepia	Sepia	F. sepia	L. sepia	-	-	Blue
4	.79	.27	-	F. purple	-	-	-	-	-	-	-	-	-
5	.88	.35	-	-	L.tan-sepia	Purple-sepia	F. rose	D. rose	F.yellow	F. sepia	-	-	F. blue
6	.90	.56	-	-	-	-	V.F. rose	-	-	-	-	-	-
7	.93	.61	-	F. white-blue	L. tan	L. tan	M. rose	Rose	F. yellow	F.sepia	-	-	-
8	.78	.58	-	B.blue-white	D. tan	L. rose	Sepia-rose	L. rose	F. yellow	F. sepia	-	-	F. blue
9	.87	.60	-	L.white-blue	-	-	-	-	-	-	-	-	-
10	.85	.65	-	F. white	L. tan	L. purple	M. rose	D. rose	F. yellow	-	-	-	F. blue
11	.85	.79	-	F. white	Tan	Tan	F. rose	F. rose	F. yellow	-	B. yellow	-	F. blue
12	.78	.75	-	F. white	-	-	F. sepia	-	-	F. sepia	-	-	-
13	.73	.74	-	-	-	L.rose-sepia	-	F. rose	F. sepia	-	-	-	-
14	.75	.90	-	F. white	-	-	-	-	-	-	-	-	-
15	.55	.67	-	F. white	Sepia-tan	L.rose to purple	-	-	-	-	-	-	F. blue
16	.68	0	Yellow-brown	Yellow-white	Yellow to tan	Tan	Yellow-orange	Yellow	F.yellow-tan	D. yellow	F. yellow to brown	Yellow	Olive-green
18	.96	0	Yellow-sepia	White	L.tan	L. tan	Yellow-tan	Tan-sepia	Yellow-tan	Sepia	-	F. brown	F. brown

Table 2b Characterisation of extra-fluorescent spots from acid hydrolized TMV infected bean leaf extracts (T) trace, (F) faint, (P) pale, (L) light, (D) deep, (ID) indeterminate.

17	.79	0	D. sepia	Light rose-white	L. tan	L. tan	Yellow-tan	Tan-sepia	Sepia	ID	-	-	T. blue
19	.86	.02	-	F. pale gr.	P. pink	P. sepia	F. sepia	D. sepia	-	-	-	-	-

these two metabolites with several of the phenolic reagent indicators suggests a complexed phenolic structure.

3. Alkaline Hydrolysis

Results obtained after alkaline hydrolysis of the ethanolic extract were similar to those obtained after acid hydrolysis(refer to acid hydrolysis).

4. Isolation and Characterisation of the Extra-Fluorescent metabolites associated with TMV-Pinto infection.

In accordance with the increased solubility of the hypersensitive spots in ether as reported by Gill(1965) for tobacco necrosis virus(TNV) infected bean leaves, di-ethyl ether extracts of TMV infected bean leaves were prepared. Chromatograms were irrigated with 15% acetic acid for 17 hrs. in the second direction, resulting in increased separation of the hypersensitive spots (See Materials and Methods). Ultraviolet examination of the chromatograms indicated the presence of 6 distinct extra-fluorescent metabolites(Fig.4). A separation of 4 extra-fluorescent metabolites was observed from metabolite 12. The chemical singularity of metabolite 11 remained uncertain as slightly differing fluorescent properties were occasionally present at the lower extremities of the spot. Fluorescence and spray reagent reactions are shown in Table 3. The fluorescent properties of several of the metabolites(11,12a,b,c,13,Fig.4) were similar to those reported by Gill(1965) in TNV infected

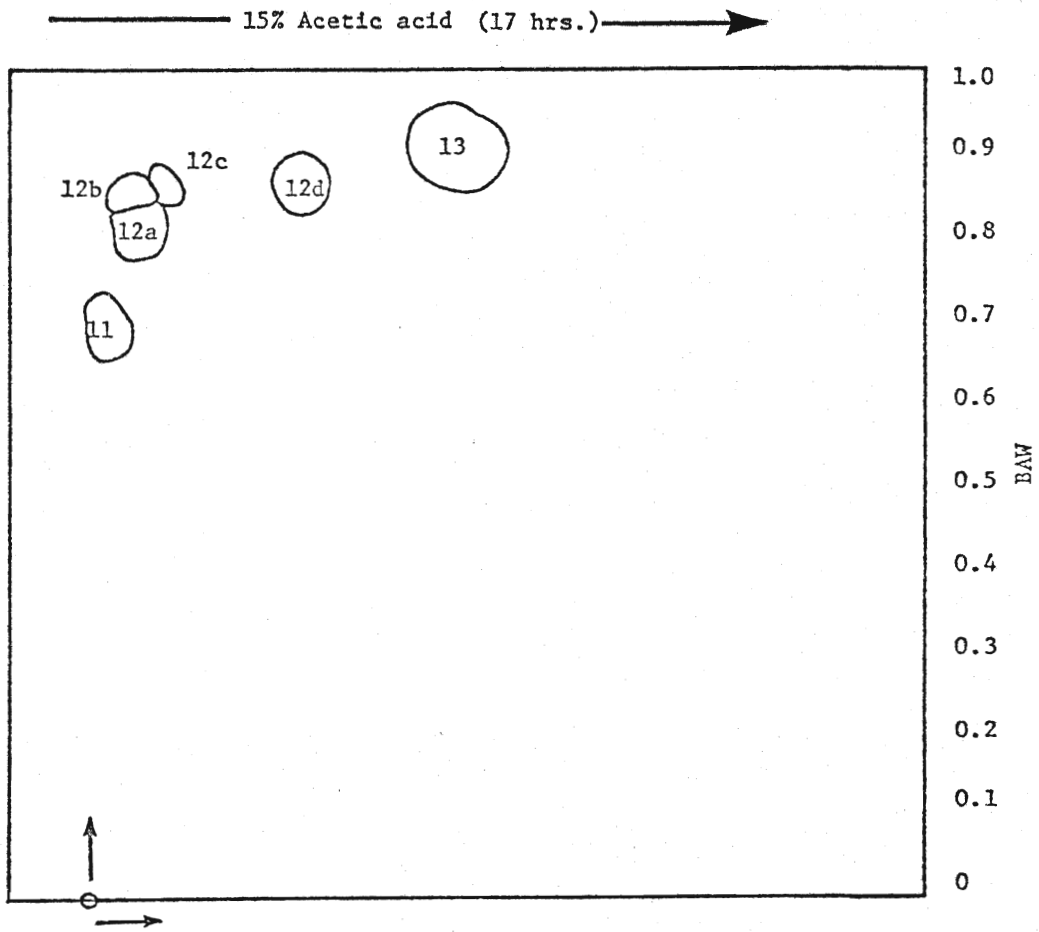


Fig. 4 Re-chromatography of the di-ethyl ether extract of hypersensitive spots of TMV infected bean leaves. Chromatograms were developed in 1-butanol:acetic acid:water, 100:27:73 (BAW) in the first direction, and in 15% acetic acid for 17 hrs. in the second direction.

Table 3 Characterisation of hypersensitive spots after development in 1-butanol:acetic acid:water 100:27:73 (BAW) in the first direction, and in 15% acetic acid for 17 hrs in the second direction. (T) trace, (F) faint, (P) pale, (B) bright, (D) deep.

No.	Colour	U.V. (2750 Å)	U.V. plus NH_4OH(vp)	U.V. plus KOH	U.V. plus 2N HCl	U.V. plus $AlCl_3$	U.V.&$AlCl_3$ plus NH_4OH	$FeCl_3 \cdot 6H_2O$ (aq)	N. Aniline	N. Aniline &20%Na_2CO_3	$AlCl_3$ (light)
11	-	purple	white to faint yellow	purple	purple	purple	B.white	-	-	-	-
12a	-	light tan	white	B. white	light tan	D. tan-brown	White	-	-	-	-
12b	-	purple	P. blue to white	purple	blue to bluish-purple	purple	White	-	-	-	-
12c	T. faint yellow	pale green to colour-less	B. orange	B. orange	Tan brown	-	Orange	-	-	-	T. faint yellow
12d	-	P. purple	turquoise to blue-green	B. blue-green	F. white	-	turquoise to green-blue	-	-	-	-
13	-	olive-green	P. green to aqua.	P. blue-green	D. blue-green	turquoise to green-blue	blue-green	P. rose	D. rose	D. rose to brown	-

Pinto bean. R_f mobilities in six solvent systems, as outlined by Reio(1958) are summarized in Fig. 5a. As a standard, a sample of chlorogenic acid was compared with the reference sample R_f value obtained by Reio(1958)(Fig.5b). No significant differences in R_f mobility were seen between the two chlorogenic acid samples.

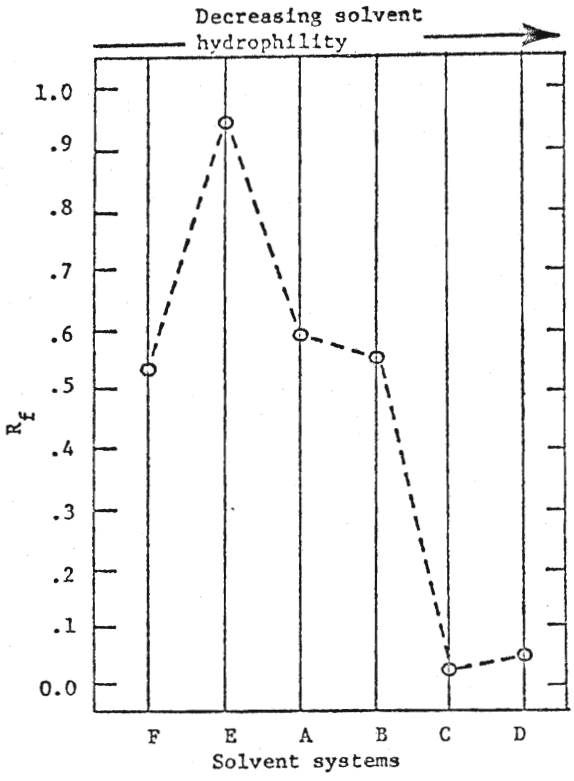
5. Examination of Extracts of Pinto Bean Infected with Bean Rust and Common Blight, for the Presence of Similar Extra-Fluorescent Metabolites.

Di-ethyl ether extracts prepared from *Uromyces phaseoli* or *Xanthomonas phaseoli* infected bean leaves failed to produce distinct extra-fluorescent metabolites corresponding with those associated with TMV infection. Trace amounts of a material similar to metabolite 11(Fig.4) however was discernible in most of the chromatograms observed.

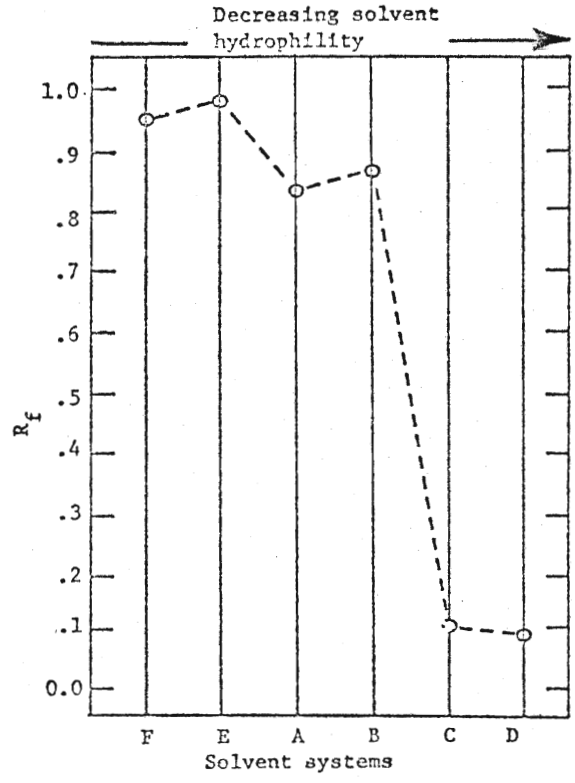
6. Time Studies

i.Time of Appearance of Extra-Fluorescent Metabolites Associated with Infection.

Primary Pinto bean leaves were inoculated with TMV to produce between 800-1500 lesions per leaf. Healthy, celite inoculated, and TMV-celite inoculated plants were maintained at 22°C. ± 2 °C. under continuous illumination (800 ft. candles) for 96 hrs. At 6 hr. intervals following inoculation, 10 gms. of leaf tissue was sampled from each set and prepared for phenol extraction. Di-ethyl ether extracts



Sample No. 1



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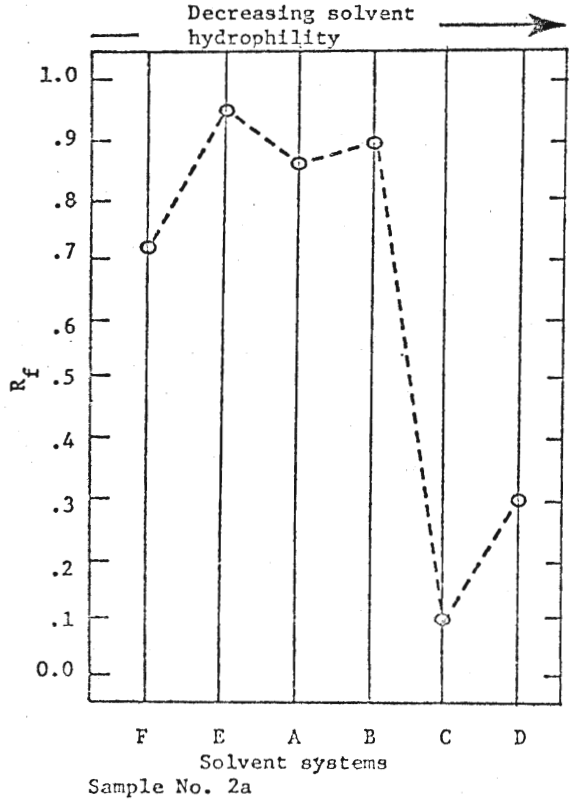

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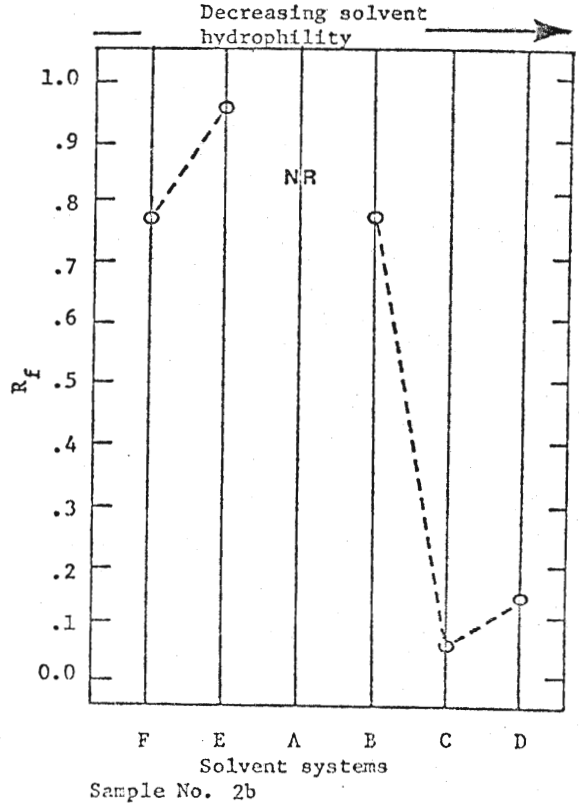

Sample No. 2b

Fig.5a R_f mobilities of hypersensitive spots in chromatographic solvent systems of Reio(1958). For solvent systems, see Materials and Methods-Analysis of extra-fluorescent metabolites.
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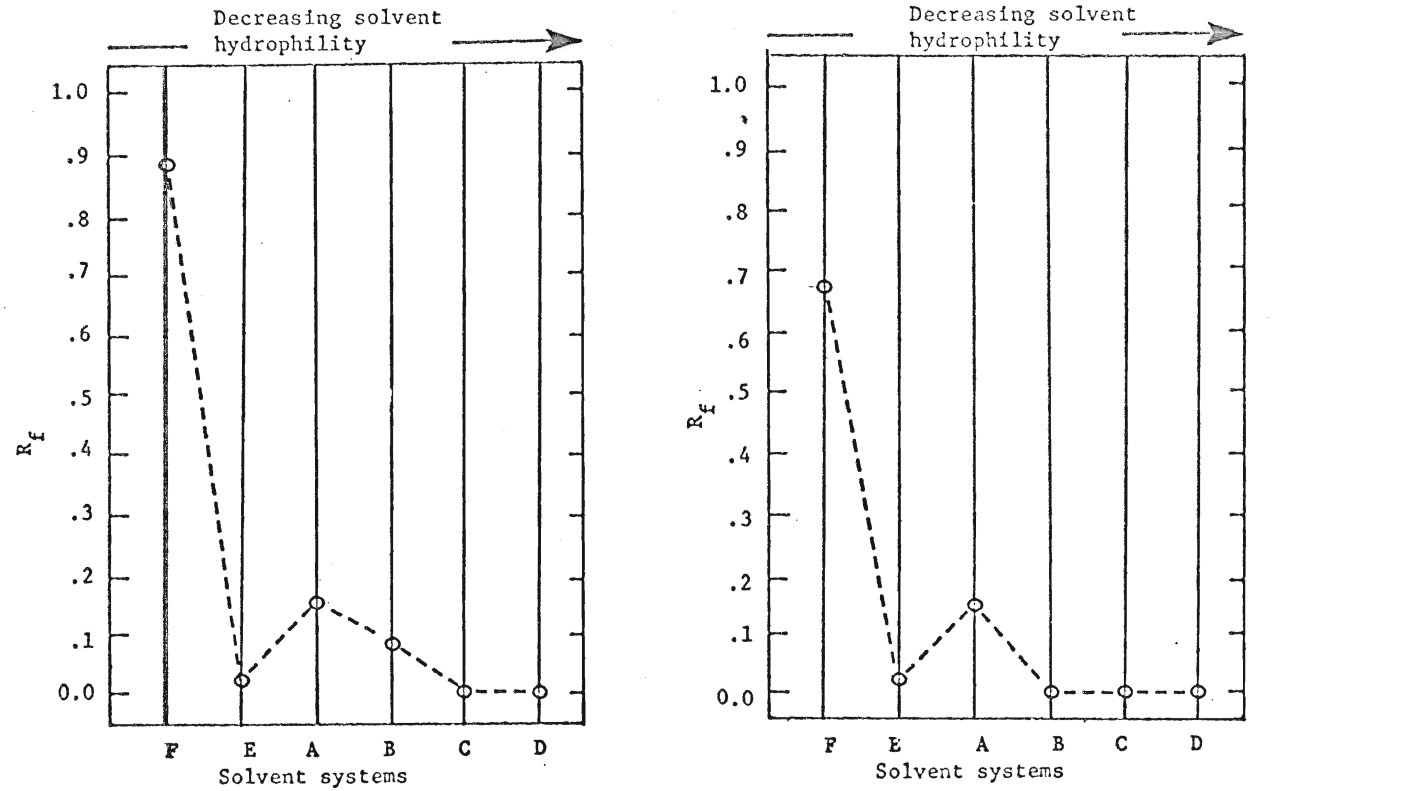


Fig.5b R_f mobilities of chlorogenic acid standard and reference sample value obtained by Reio(1958).

prepared from the ethanolic unhydrolized phenol extracts were chromatographed in BAW(100:27:73) and irrigated for 20 hrs. in 15% acetic acid in the second direction and were examined under ultraviolet light.

The first of the extra-fluorescent metabolites appeared 27 hrs. after inoculation(Table 4a). This metabolite(11)was was detected in trace amounts in TMV-celite inoculated leaves and increased appreciably with the expression of local lesions 30 hrs. after inoculation. Metabolites 12a,b,c,d and 13 appeared at 30 hrs., coincident with lesion expression. No extra-fluorescent metabolites were noted in healthy or celite inoculated leaves over the test period. Fluorescent microscope observations of infected leaf tissue revealed isolated occurrences of fluorescent metabolites approximately 16 hrs. after viral inoculation. Such fluorescence however, was present only at trace levels. Attempts to record this on film were unsuccessful.

ii. Time of Appearance of Extra-Fluorescent Metabolites Associated with Wounding.

Light mechanical injury was induced on primary Pinto bean leaves by light abrasion of each leaf with emery paper grade number 600. Severe mechanical abrasion was effected by applying slightly increased pressure on the emery paper during abrasion. Leaves were briefly rinsed in running water and incubated at 22° C. ± 2 °C. under continuous illumination. Sampling methods and chromatographic analysis are as described

	Time (hrs) following TMV inoculation										
	0	6	12	18	24	27	30	33	36	72	96
Healthy (22°C)	-	-	-	-	-	-	-	-	-	-	-
Celite wounded (22°C)	-	-	-	-	-	-	-	-	-	-	-
Celite and TMV (22°C)	-	-	-	-	-	11(T)	12abcd 11,13	12abcd 11,13	12abcd 11,13	12abcd 11,13	12abcd 11,13

Table 4a Appearance of hypersensitive metabolites in TMV infected bean leaves at 22°C. (T) trace.

Abrasion	(light)	-	-	I.D.	11(T)	11(T)	11(T)	11(T)	11(T)	11(T)	11(T)	11(T)
Abrasion	(severe)	-	-	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)	11(T) 12(T)

Table 4b Appearance of hypersensitive metabolites in bean leaves after light and heavy abrasion. (T) trace.

Healthy	(37°C)	-	-	-	-	-	-	-	-	-	-	-
Celite wounded	(37°C)	-	-	-	-	-	-	-	-	-	-	-
Celite and TMV	(37°C)	-	-	-	-	-	-	-	-	-	-	-
Celite and TMV	(22°C)	-	-	-	-	-	11(T)	12abcd 11,13	12abcd 11,13	12abcd 11,13	12abcd 11,13	12abcd 11,13

Table 4c Appearance of hypersensitive metabolites in TMV infected bean leaves at 37°C. (T) trace, (ID) indeterminate.

Healthy	(&.05M AA)	-	-	-	-	-	-	-	-	-	-	-
Celite wound	(&.05M AA)	-	-	-	-	-	-	-	-	-	-	-
Celite & TMV	(&.05M AA)	-	-	-	-	-	-	-	-	-	-	-
Celite & TMV	(No AA)	-	-	-	-	-	11(T)	11(T)	12abcd 11,13	12abcd 11,13	12abcd 11,13	12abcd 11,13

Table 4d Appearance of hypersensitive metabolites in TMV infected bean leaves previously infiltrated with ascorbic acid (AA).

above.

Light mechanical abrasion produced no visible necrotic areas. However abrasion of this type was found to induce trace amounts of metabolite 11 at 18 hrs.after injury(Table 4b). Severe abrasion resulted in the appearance of large necrotic areas on the leaf surface approximately 6 to 12 hrs. after abrasion (Figs 6,7). The appearance of metabolites 11 and 12 was noted 12 hrs. after injury. Acid hydrolysis of the severe abrasion extract resulted in the appearance 12 hrs. after injury of a pale yellow band, fluorescing yellow in ultraviolet light. The properties of this metabolite are similar to those recorded for metabolite 17(Acid Hydrolysis- TMV-celite inoculation,Fig.3).

iii. Appearance of Extra-Fluorescent Metabolites on Necrotic Suppression at Elevated Temperatures.

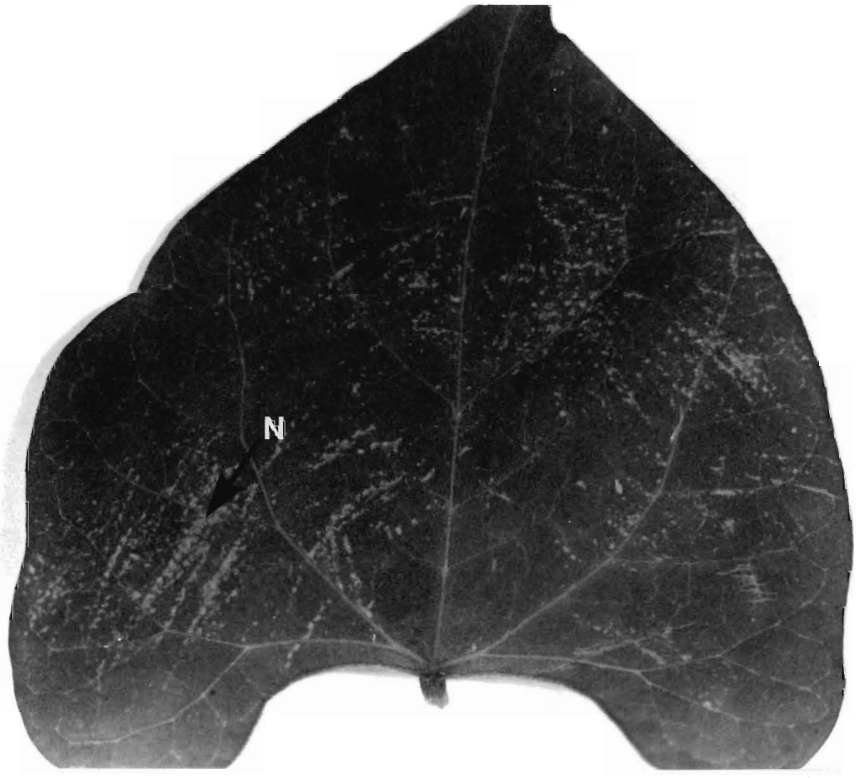
Healthy, celite inoculated, and TMV-celite inoculated Pinto bean leaves were floated on water baths incubated at 37°C. ± 2°C. Control leaves were incubated at 22°C. ± 2°C. A continuous photoperiod of 800 ft. candles was maintained over a 96 hr. period. Sampling and chromatographic analysis are as described previously.

Leaves incubated at 37°C. developed limited necrosis. Viral lesions were not recognizable although some necrotic areas were present along vein margins in TMV-celite inoculated leaves. No appreciable levels of extra-fluorescent metabolites were

Fig. 6 Effect of severe mechanical abrasion on Pinto bean leaf tissue (X1.5)

(N) necrotic area

Fig. 7 Extensive necrotic area (➤) in severly damaged Pinto bean leaf tissue (X15)


N

detected on paper chromatograms(Table 4c) although acid hydrolysis revealed intermittently occurring trace amounts of a faint yellow coloured band, fluorescing yellow in ultraviolet light, in the TMV-celite assay preparations. Formation of extra-fluorescent metabolites coincided with lesion expression at 30 hrs. in control leaves incubated at 22°C.

iv. Appearance of Extra-Fluorescent Metabolites on Necrotic Suppression Associated with Ascorbic Acid Infiltration.

From previous studies(Stobbs,1973) 0.05M ascorbic acid inhibited necrotic symptom expression in TMV infected Pinto bean leaves without causing appreciable leaf damage. Leaves were vacuum infiltrated with 0.05M ascorbate and floated on the ascorbate solution. Control leaves were water infiltrated and accordingly floated on water baths. Leaves were maintained under constant illumination (800 ft. candles) at 22° C. ± 2 °C. Three hours after vacuum infiltration the leaves were inoculated with TMV, rubbed with celite,or water alone and returned to their respective baths and incubated for 96 hrs. Sampling and chromatographic analysis are as described previously.

Leaves infiltrated with ascorbate solution were symptomless. No extra-fluorescent metabolites were detected in any of the ascorbate infiltrated leaves (Table 4d). Extra-fluorescent metabolites appeared in water-infiltrated TMV-celite inoculated leaves coincident with the appearance of lesions.

v. Association of Extra-Fluorescent Metabolites with Necrotic Suppression.

Elevated temperatures and exogenous application of various reducing agents to various hypersensitive tissue has been shown to result in necrotic suppression. On removal of the applied factor, necrosis rapidly occurs. The association of extra-fluorescent metabolites with recurrent necrosis was questionned.

a. Studies on temperature transposition

Primary Pinto bean leaves subjected to pre-darkening treatment were inoculated with TMV and floated on water baths maintained at 37 °C. and 22°C. under continuous illumination. Following incubation for 60 hrs., samples of leaves from each temperature were prepared for chromatography. The remaining leaves were briefly rinsed in running water and transferred to water baths maintained at 22 °C. and incubated for and additional 24 hrs. under similar light conditions. Samples from the two treatments were subsequently prepared for chromatography(Fig.8).

Chromatographic data is presented in Table 5a. Slight necrosis was noted in TMV-celite inoculated leaves incubated at 37 °C. for 60 hrs. although necrotic tissue was localised along vein margins. Lesions developing on leaves incubated at 37°C. and returned to 22°C.were slightly larger than those lesions appearing on leaves incubated at 22 °C. alone. On treatment of the leaves

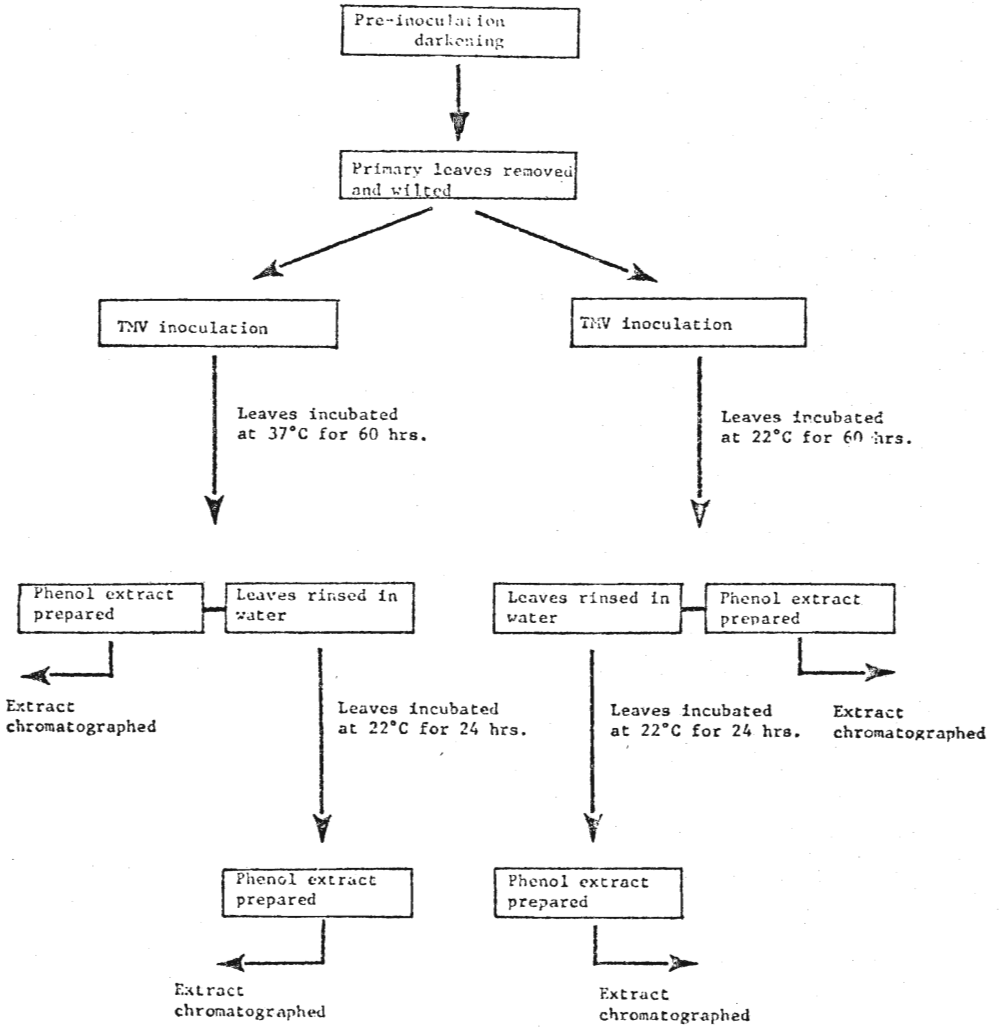


Fig.8 Procedure for sampling of extracts in temperature transposition studies.

Table 5a Appearance of hypersensitive metabolites in temperature transposition studies.

Extract Preparation	Healthy	Celite inoc.	TMV-Celite	Presence(+)or Absence (-)of visible necrosis
Leaves incubated at 37°C for 60 hrs.	-	-	-	-
Leaves incubated at 22°C for 60 hrs.	-	-	11,13 12abcd	+
Leaves incubated at 37°C for 60 hrs and returned to 22°C for 24 hrs.	-	-	11,13 12abcd	+
Leaves incubated at 22°C for 60 hrs. and returned to 22°C for 24 hrs.	-	-	11,13 12abcd	+

Table 5b Appearance of hypersensitive metabolites in ascorbate studies

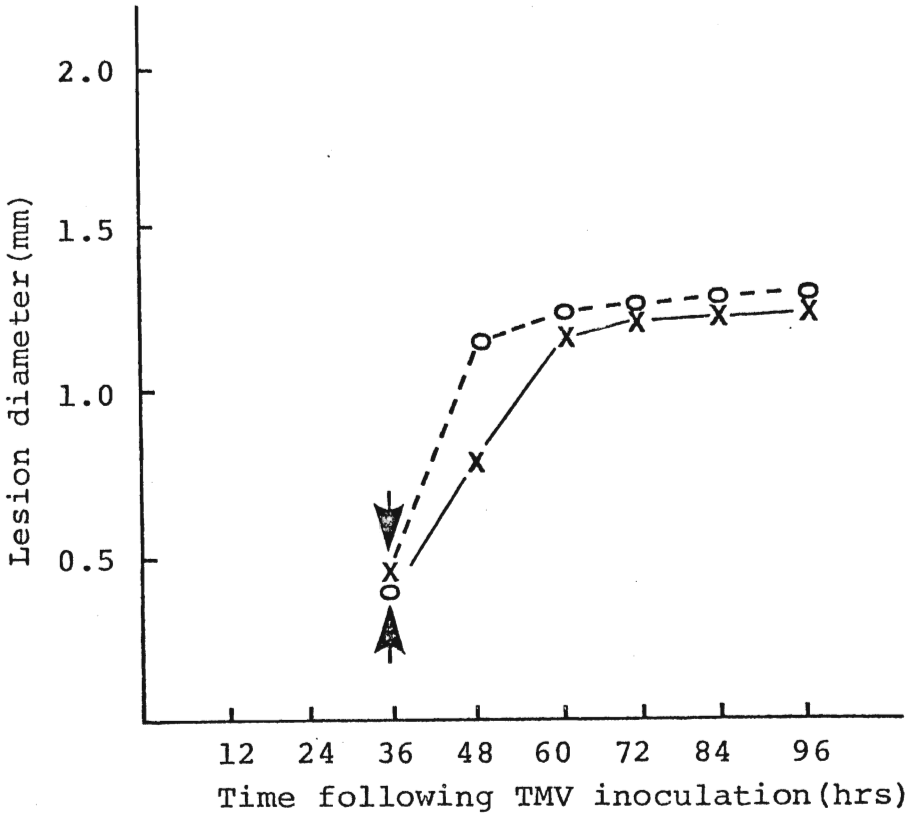
Extract Preparation	Healthy	Celite inoc.	TMV-Celite	Presence(+)or Absence (-)of visible necrosis
Leaves incubated with ascorbate for 60 hrs.	-	-	-	-
Leaves incubated in water bath for 60 hrs.	-	-	11,13 12abcd	+
Leaves incubated in ascorbate for 60 hrs. and returned to water bath for 24 hrs.	-	-	11,13 12abcd	+
Leaves incubated in water bath for 60 hrs. and returned to water bath for 24 hrs.	-	-	11,13 12abcd	+

for 2 min. in a 50°C. water bath, lesions were found to rapidly enlarge(Fig.9). Appearance of extra-fluorescent metabolites was coincident with lesion expression. A faint yellow substance, fluorescing yellow in ultraviolet light, was detected in chromatograms obtained from extracts exposed to 37°C. temperatures. The fluorescent property of this substance was similar to that of metabolite 17 obtained in acid hydrolysis(Fig.3-Acid hydrolysis). Chromatographic extracts prepared from leaves incubated at 37°C. for 84 hrs. similarly revealed traces of this compound.

b. Ascorbate studies

Detached Pinto bean primary leaves were vacuum infiltrated with 0.05M ascorbic acid three hours prior to TMV inoculation, and floated on ascorbate baths. Control leaves were water infiltrated and floated on water baths. Following TMV inoculation, the leaves were incubated under continuous illumination for 60 hrs. at 22°C. in their respective baths. Following incubation, samples of leaves from each bath were prepared for chromatography. The remaining leaves were rinsed in running water and transferred to water baths at 22°C. for an additional 24 hrs. Samples from each bath were subsequently prepared for chromatography(Fig.10).

Chromatographic data is presented in Table 5b. Leaves incubated in ascorbate solutions were symptomless. Lesions on



X——X Lesion expansion in Pinto bean leaves raised at 22° C

o---o Lesion expansion in Pinto bean leaves raised at 22° C following heat treatment for 2 min at 50 C° at 36 hrs.

◄ ► Time of lesion appearance

Values represent mean lesion diameter. Standard error of the mean <0.02 Each point represents ten measurements, one leaf per condition. The same lesions were measured for each subsequent value.

Fig. 9 Effect of post-lesion heat treatment on lesion expansion in TMV infected Pinto leaves.

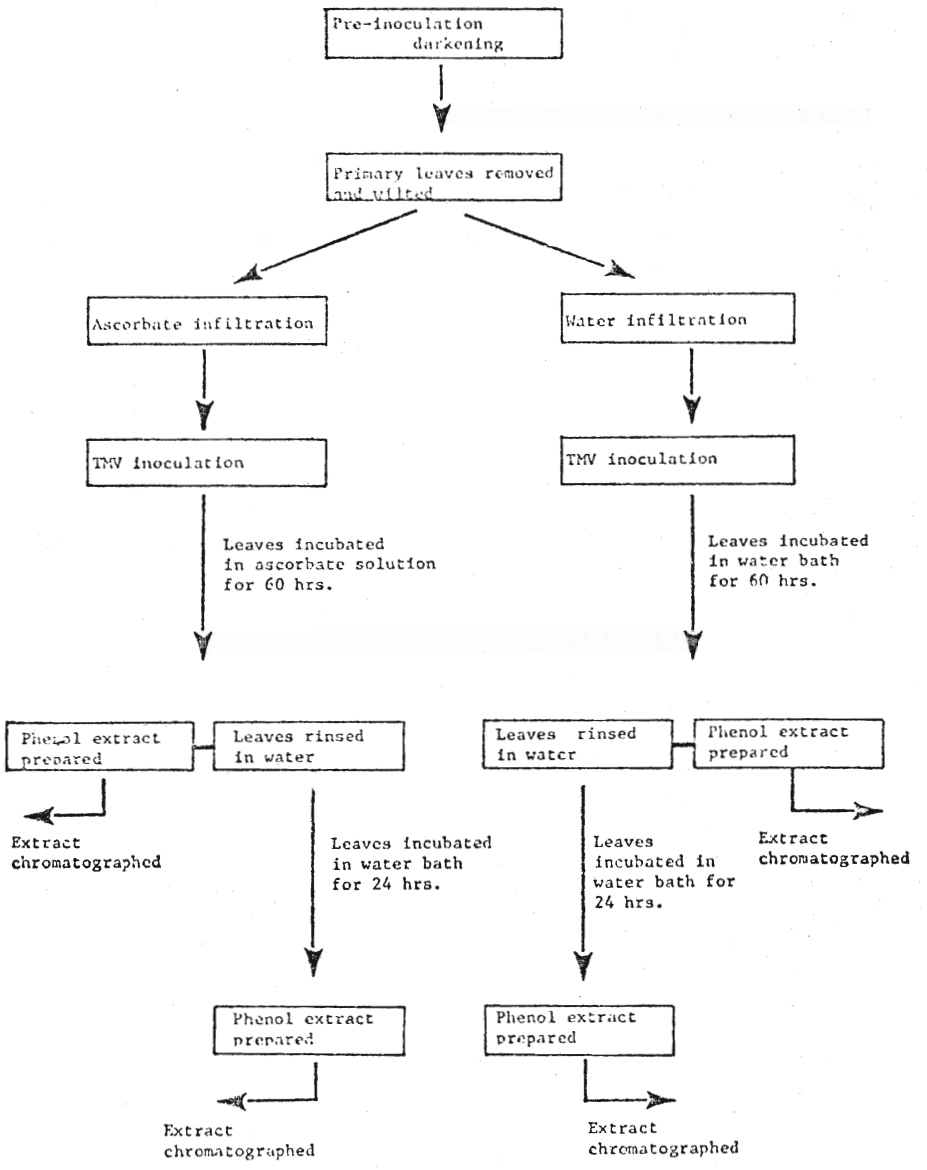


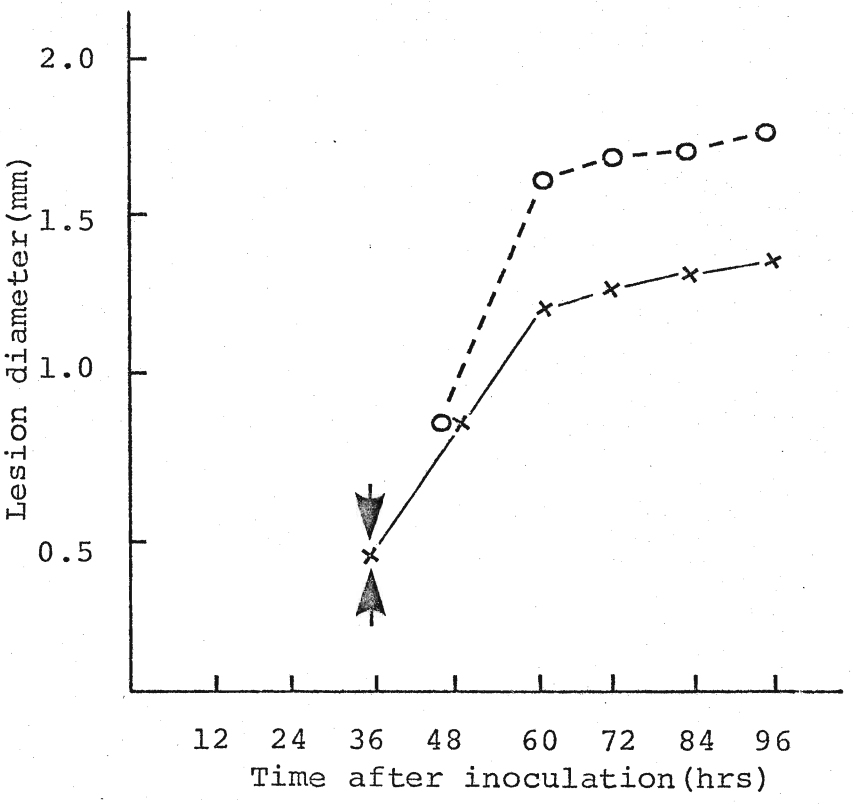
Fig.10 Procedure for sampling of extracts in ascorbate studies

leaves pre-incubated with ascorbate on returning to the water bath, were larger than those lesions incubated in the water bath alone(Fig.11-14). Lesions appeared 12 hrs. after the transfer of the leaves from the ascorbate solution to the water baths. Tissue darkening along veinal tissue was recorded 48 hrs. following the transfer(Fig.15). The appearance of extra-fluorescent metabolites was noted to be concomitant with lesion expression. Maximum lesion necrosis was not apparent until 30 hrs. after the change. Chromatographic extracts prepared from leaves incubated in ascorbate for 84 hrs. revealed no traces of any extra-fluorescent metabolites.

vi. Determination of the Time of Necrotic Induction

Ascorbate treatments reduce or prevent necrotic symptom development(Farkas et al,1960). High levels of ascorbate if applied for short periods of time during the infection process may delay the time of lesion appearance if application is prior to necrotic induction. Application of ascorbate after this point may not alter the time of lesion appearance. Such *shock ascorbate infiltrations* would allow the determination of the time of irreversible necrosis in the infection process.

With this objective, Pinto bean plants following germination, were transplanted to 7.6cm. clay pots containing vermiculite, and fed with nutrient solution until the primary leaves were developed. Plants were selected having uniform primary leaves



o---o Lesion expansion in ascorbate infiltrated Pinto leaves transferred to water bath 36 hrs after inoculation.

x——x Lesion expansion in water infiltrated Pinto leaves.

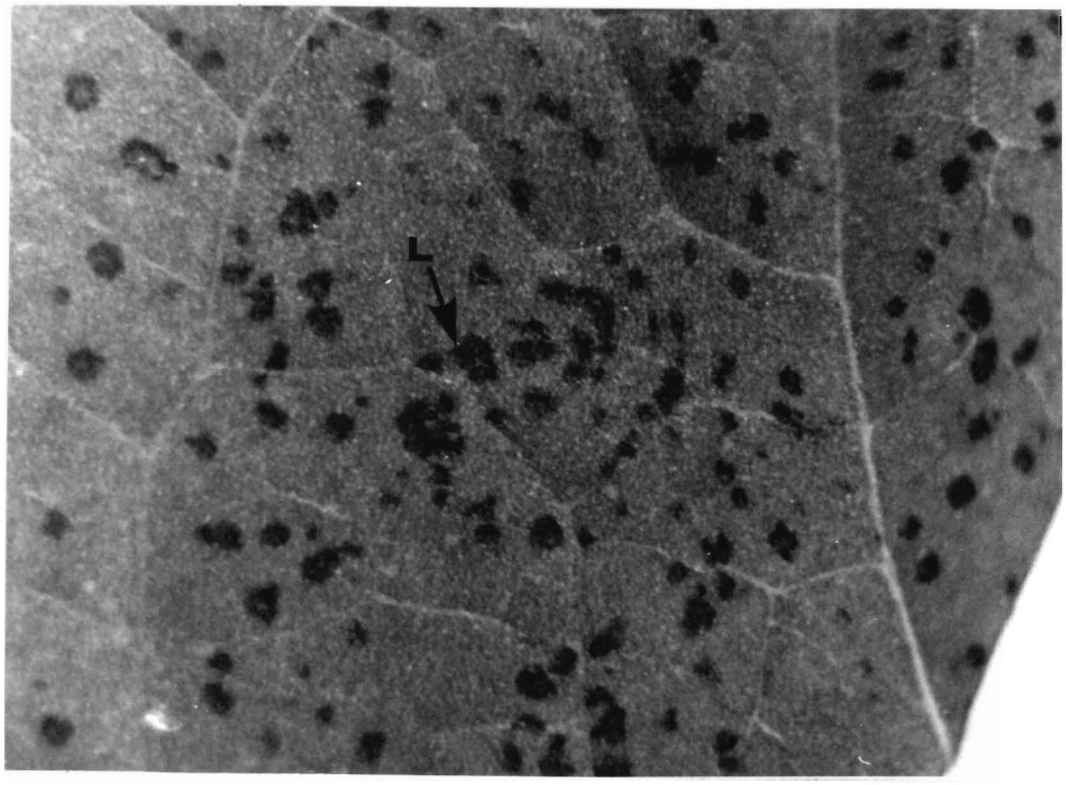
◀ ▶ Time of lesion expression in water infiltrated leaves.

Values represent mean lesion diameter. Standard error of the mean <0.02 Each point represents ten measurements, one leaf per condition. The same lesions were measured for each subsequent value.

Fig. 11 Effect of ascorbate infiltration on lesion expansion in TMV infected Pinto leaves.

Fig. 12 Lesions(L) on TMV inoculated Pinto bean leaves 36 hrs. after inoculation (X15)

Fig. 13 Enlarged lesions(EL) on TMV inoculated bean leaves following post-infiltration of ascorbate over a 24 hr. period initiated on lesion expression. Lesions developed 12 hrs. following termination of the ascorbate treatment (X15)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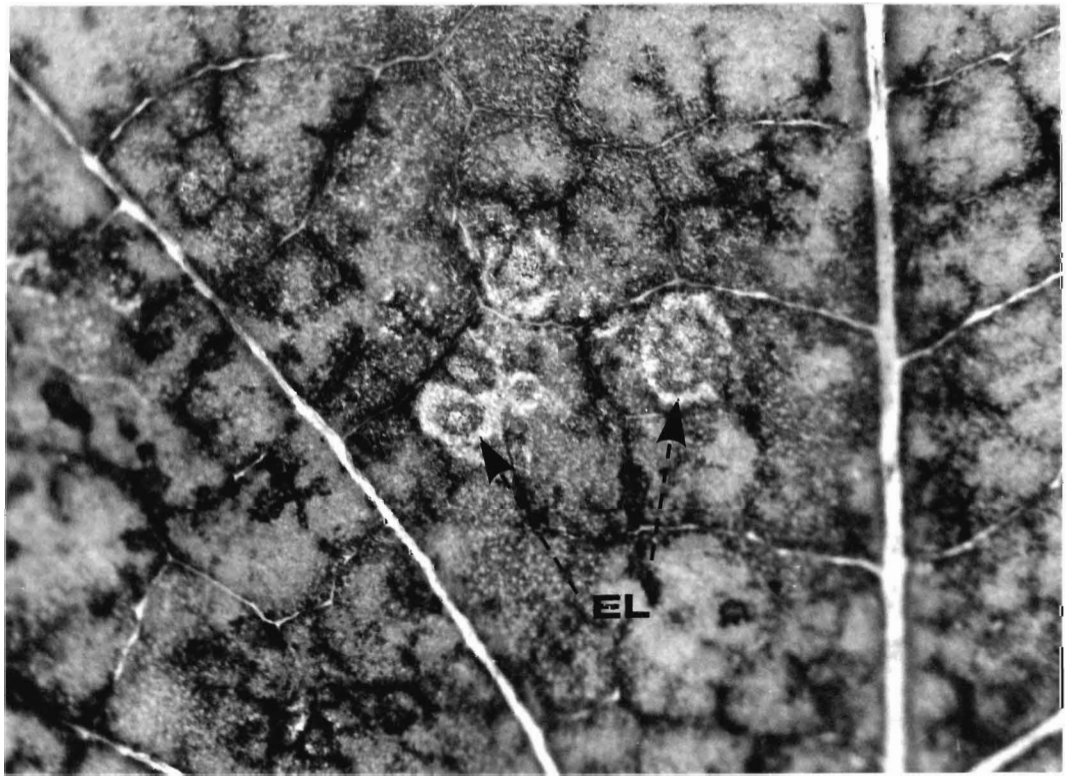
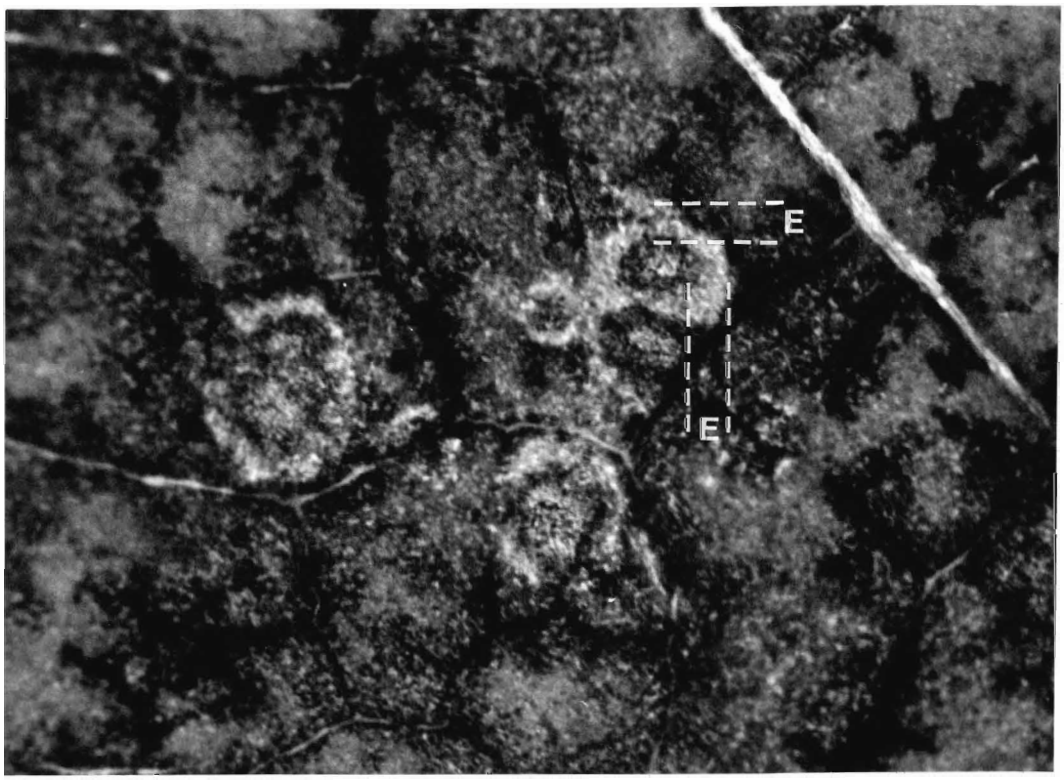

EL

Fig. 14 Enlarged TMV lesions developing on post ascorbate infiltrated leaves (X 30)

(E) zone of lesion enlargement

Fig. 15 Areas of veinal darkening(D) in post ascorbate infiltrated leaves (X30)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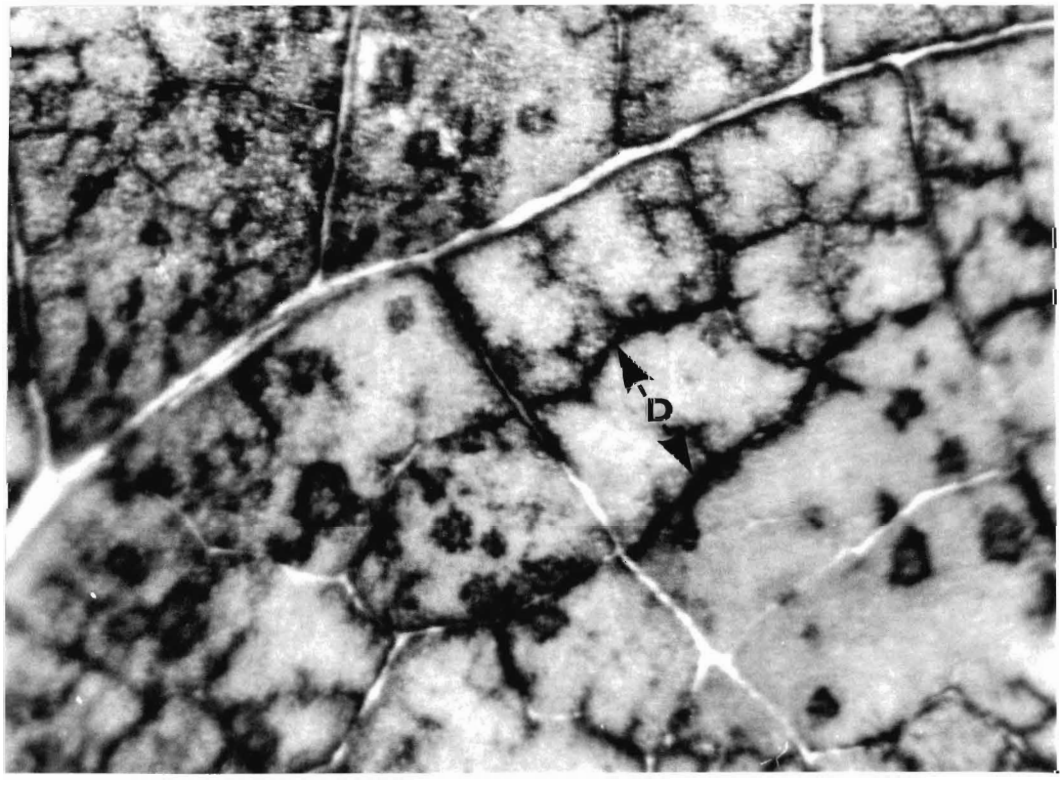

D

of approximately the same size. Primary leaves were inoculated with TMV to produce approximately 500 to 800 lesions per leaf. Following inoculation, two samples were taken, one of which was vacuum infiltrated with ascorbate, the other vacuum infiltrated with water. The leaves were briefly rinsed in running water, and the plants repotted in vermiculite. Plants were returned to the growth chamber and received continuous illumination(800 ft. candles). At succeeding 3 hr. intervals, plants were infiltrated similarly and returned to the growth chamber. The time of lesion appearance was noted for all the test plants.

The effect of ascorbate infiltration on the time of lesion expression was examined in two experiments. In experiment 1, ascorbate produced a delay in lesion appearance if applied within the first 18 hrs. after inoculation(Fig.16). The times of lesion appearance in subsequent ascorbate infiltrations closely approximated the time of lesion formation of leaves infiltrated with ascorbate at the time of inoculation. A slight delay in lesion expression occurred in water infiltrated leaves 6 hrs. after inoculation; further changes were not however significantly apparent. Lesions appearing on leaves infiltrated with ascorbate prior to 18 hrs. were slightly smaller than lesions appearing on leaves infiltrated with ascorbate at 27 hrs.,or lesions on water infiltrated controls. Non-infiltrated leaves developed lesions at 30 hrs. after inoculation. Similar results are apparent in experiment 2, with maximum

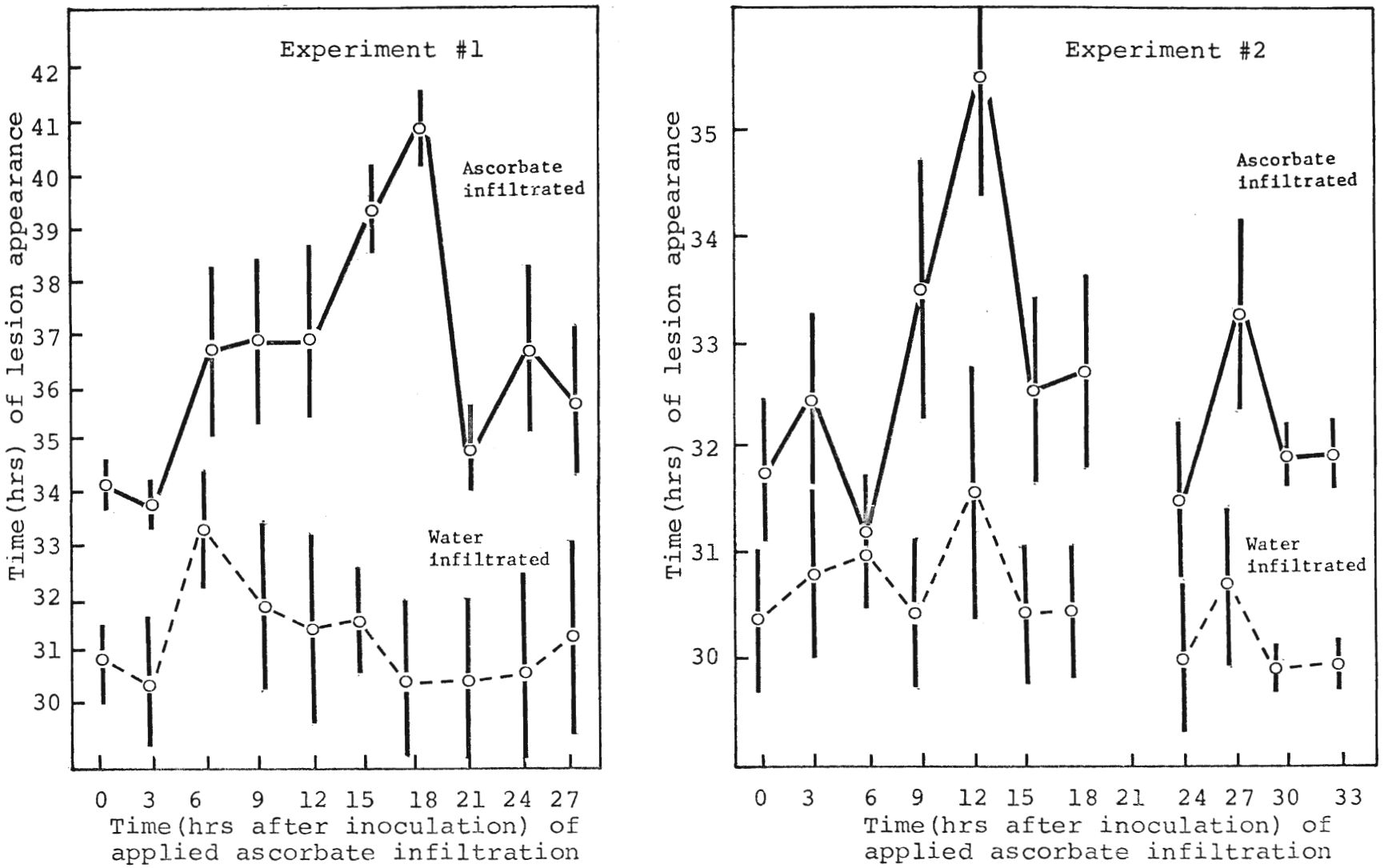


Fig. 16 Determination of the time of necrotic induction in TMV infected Pinto bean leaves as determined by ascorbate infiltrations.
—o— represents mean time ± standard deviation n=10

delayment in lesion appearance occurring at 12 hrs.(Fig.16).

B. HISTOLOGICAL OBSERVATIONS

1. Light Microscopy of Healthy and TMV Infected Tissue

Pinto bean leaf tissue examined under the light microscope exhibited normal cuboidal cells, the underlying rectangular palisade parenchyma cells being lined peripherally with chloroplasts(Fig.17). A less compact spongy mesophyll parenchymatous layer contained numerous air spaces. Celite inoculated leaves exhibited similar structure with occasional collapsed epidermal cells.

Sections of TMV-celite inoculated tissue indicated areas of cellular necrosis corresponding to the lesion position (Fig.18). This necrotic area lacked a distinct epidermal layer, where the existing cells exhibited abnormal concavity. No cellular detail could be distinquished. Chloroplasts were not apparent. A semi-necrotic zone surrounding the necrotic lesion centre, was characterised by the opacity of the cell wall and the presence of chloroplasts in the parenchyma. No change in the lower epidermis was noticeable.

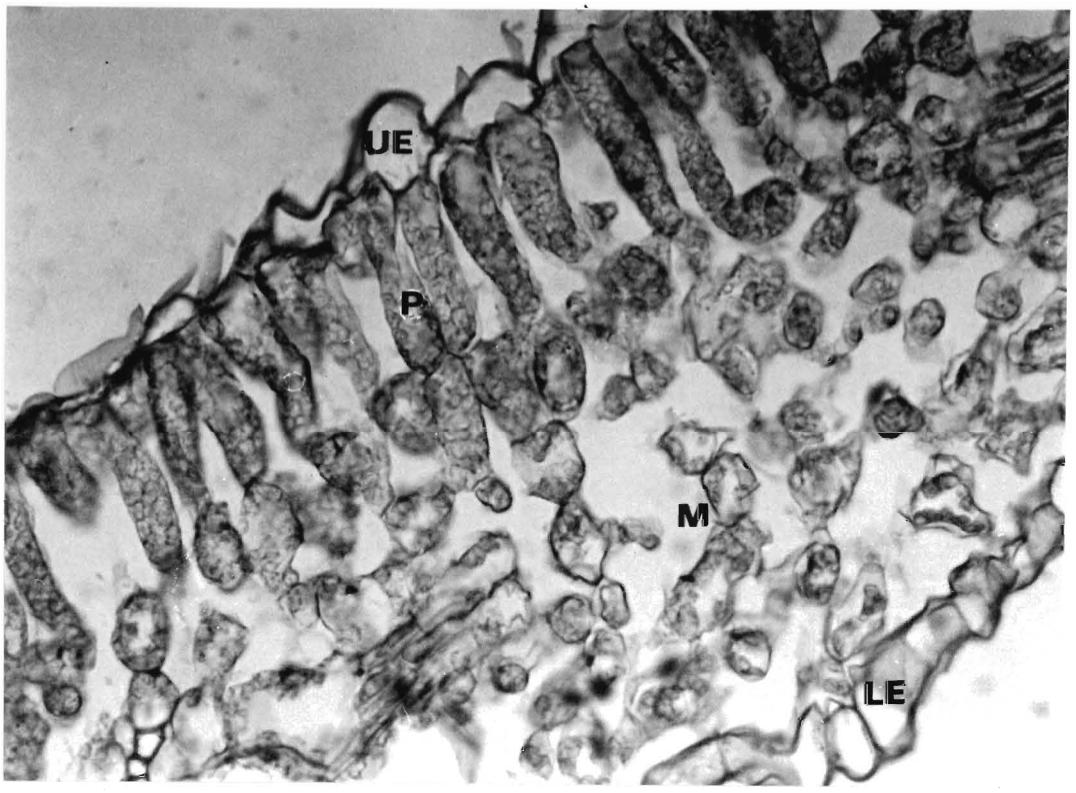
2. Fluorescent Microscopy of Surficial Fluorescent Deposits Surrounding Leaf Tissue

Pale yellow-green fluorescing halo rings extended

Fig. 17 Cross section of healthy Pinto bean leaf tissue (X800)

(UE) upper epidermis
(P) palisade cells
(M) mesophyll cells
(LE) lower epidermis

Fig. 18 Cross section through a lesion in TMV infected Pinto bean leaf tissue. Arrows indicate lesion extremities (X800)


UE
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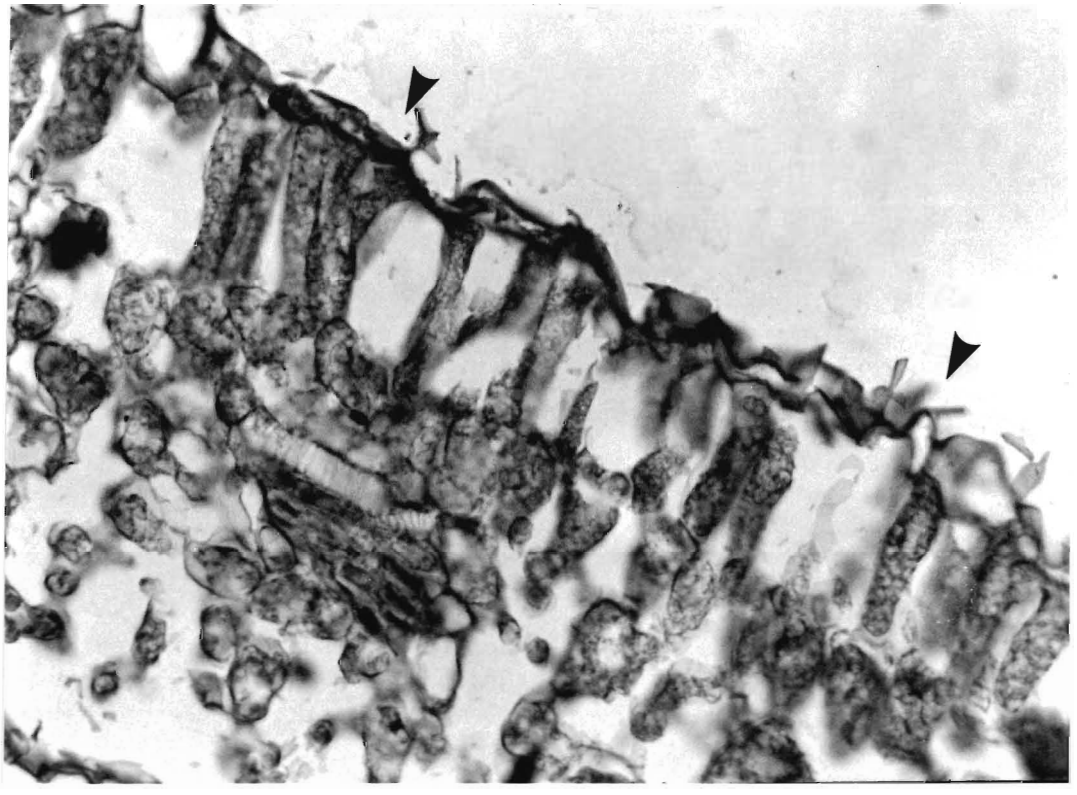


4 to 5 mm. beyond the necrotic lesion centre(Fig.19). Fluorescence was most intense in cells immediately bordering the visible necrotic tissue. Fluorescence diminished as the distance from the lesion increased. Mechanically damaged leaf tissue contained fluorescent deposits within several minutes of injury. Such fluorescence was confined to the injured tissue. No corresponding fluorescence was observed in healthy leaf tissue.

3. Callose Studies

i. Occurrence of Callose in TMV infected Pinto Bean Leaf Tissue

Healthy, celite inoculated, and TMV-celite inoculated bean leaves were sampled at 3 hr. intervals over a 33 hr. period. Additional samplings were taken at 45 hrs. and 78 hrs. after inoculation. Tissues for callose assay were prepared and stained as described (See Materials and Methods-Callose stain). Results are outlined in Table 6. Lesions on TMV-celite inoculated leaves were apparent 33 hrs. after inoculation. Aniline blue was found most valuable in callose detection.

Sections prepared during the early stages of the infection process(0-18 hrs.) in TMV-celite inoculated leaves revealed trace amounts of callose throughout the sample tissue. These deposits were generally restricted to single epidermal or palisade cells. Similar scattered callose deposits in celite wounded tissue

Fig. 19 Fluorescent micrograph of the surface of a lesion illustrating the deposition of fluorescent metabolites around the lesion perimeter (X150)

(N) zone of visible necrosis
(F) fluorescent halo deposition

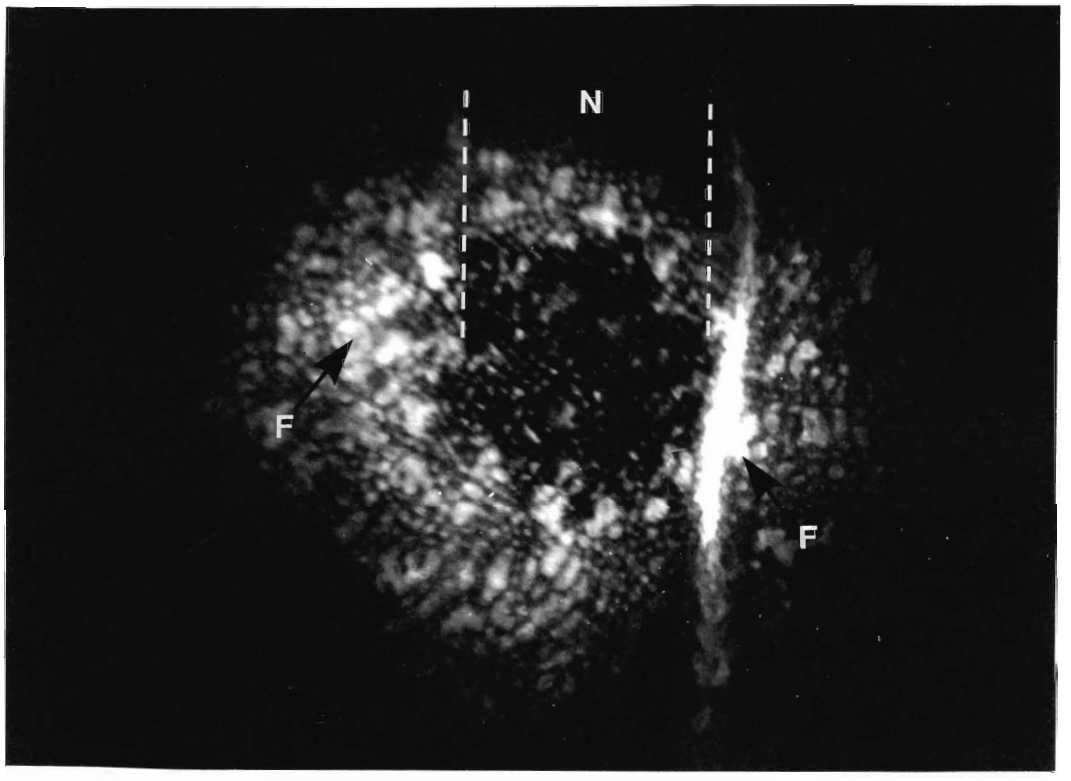

N
F
F

Table 6 Time of callose formation as determined by light and fluorescent stains (R) resorcinol, (L) lacmoid blue,(A) aniline blue fluorochrome.

Id: indeterminate
+ : callose present
- : no callose detected

Time(hrs) following TMV inoculation	Healthy			Celite			TMV & Celite		
	R	L	A	R	L	A	R	L	A
0	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	Id
30	-	-	-	-	-	-	+	+	+
33	-	-	-	-	-	-	+	+	+

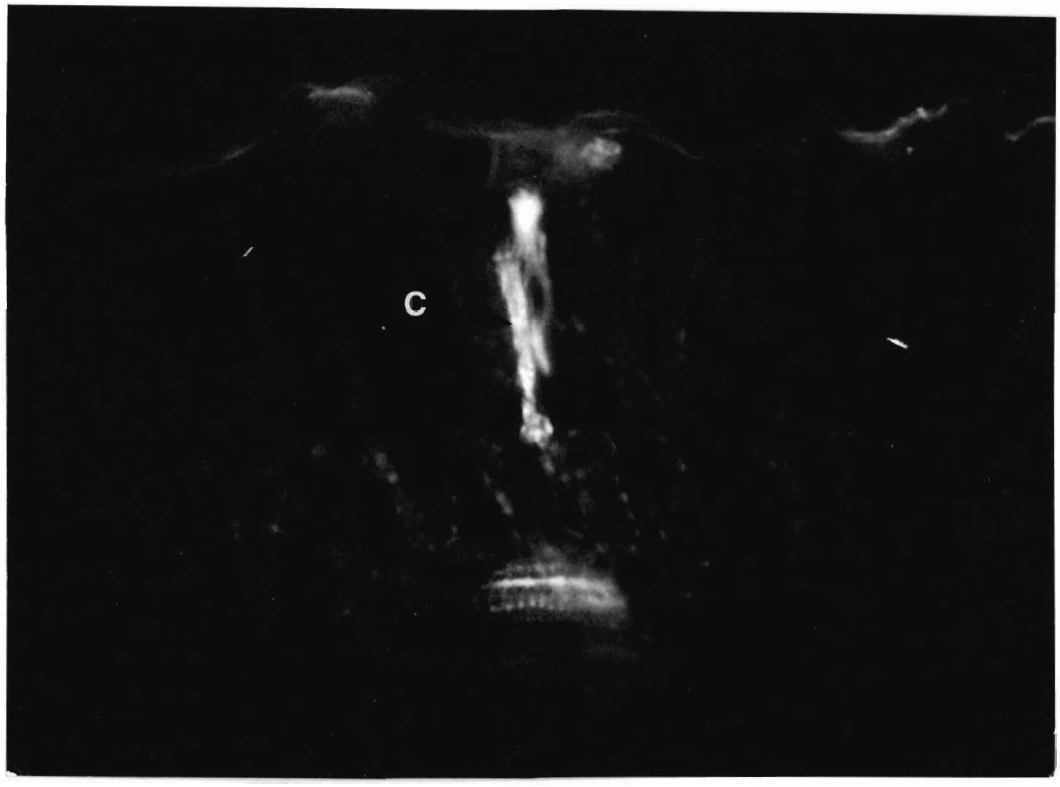
suggest that callose formation may be associated with a generalised host response to injury. Significant callose depositions were not found in healthy or celite wounded tissue during the study period.

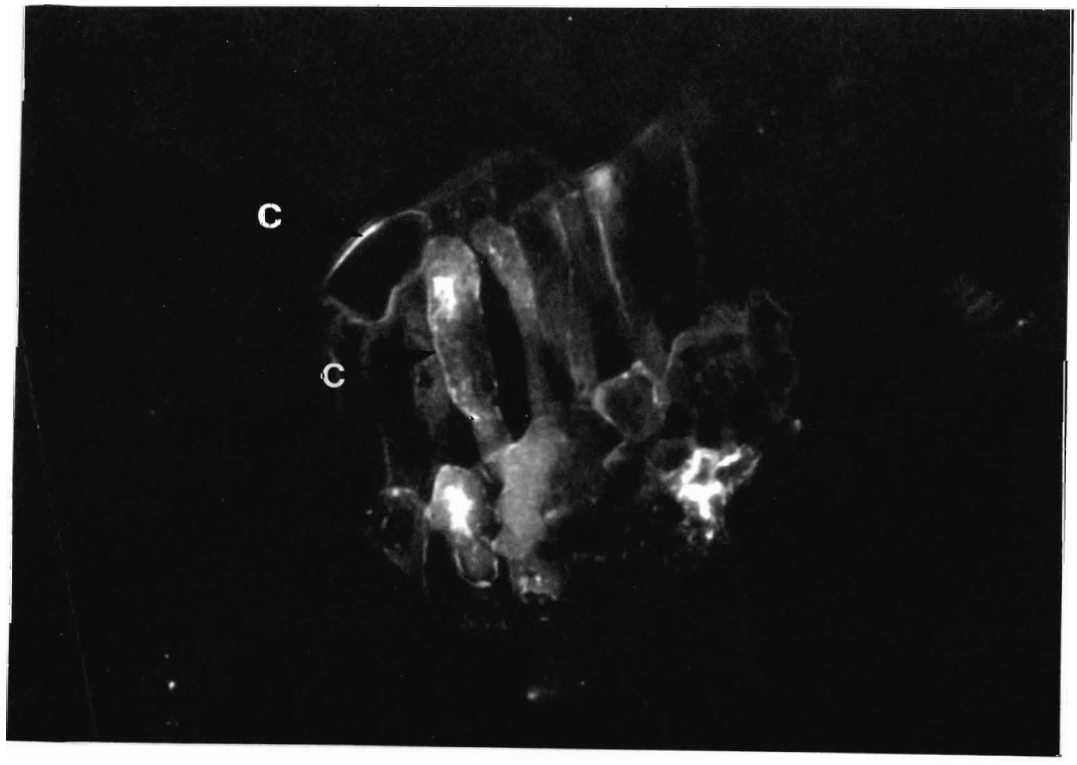
Callose was first observed in significant levels at 27 hrs. in TMV-celite inoculated leaves. Such deposits, generally confined to several neighbouring cells, were seen in the upper epidermal and palisade layer of the leaf(Fig.20). Many of these cells were in a semi-collapsed state. On lesion appearance at 30 hrs. after inoculation, lesion tissue was characterised by callose fluorescence in epidermal, palisade, and upper mesophyll tissue(Fig.21). Maximum lateral callose spread was noted at 48 hrs(Fig.22). Areas of cellular collapse were most predominant at the lesion centre. Epidermal cells at the lesion centre were ruptured, and underlying palisade cells were severly constricted. Intense callose fluorescence was noted in cells retaining structural integrity. At 72 hrs. no increase in lesion size was apparent, however most epidermal and palisade cells that comprised the lesion had collapsed producing a distinct concavity in the upper leaf surface(Fig.23).

Callose deposits were generally restricted to the upper epidermis, palisade and upper mesophyll tissues(Fig.24). No callose deposition was observed in the lower mesophilic cells and lower epidermis. Fluorescence was limited to those cells contained within the lesion or directly adjacent to it. Such cells, on examining under the light microscope, were seen to be

Fig. 20 Fluorescent micrograph showing callose deposition(white areas) in Pinto bean leaf 27 hrs. after TMV inoculation (X800) Stain: Aniline blue

Fig. 21 Fluorescent micrograph showing callose deposition in Pinto bean leaf 30 hrs. after TMV inoculation (X800) Stain: Aniline blue


C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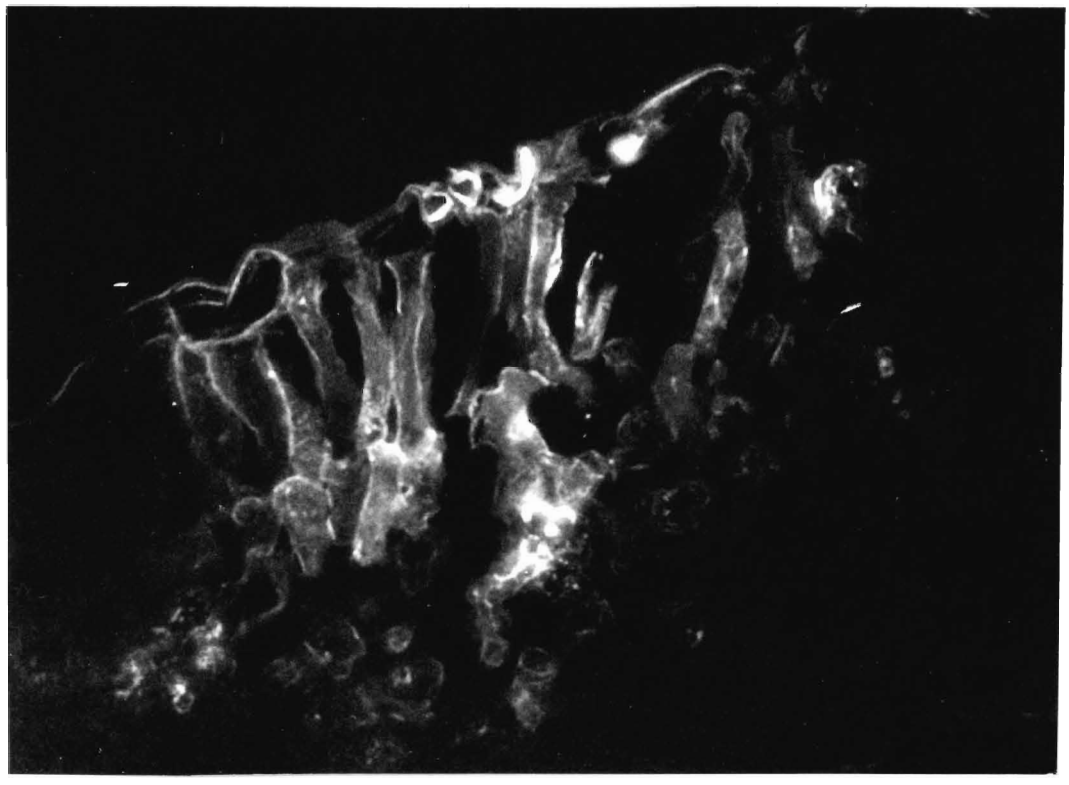


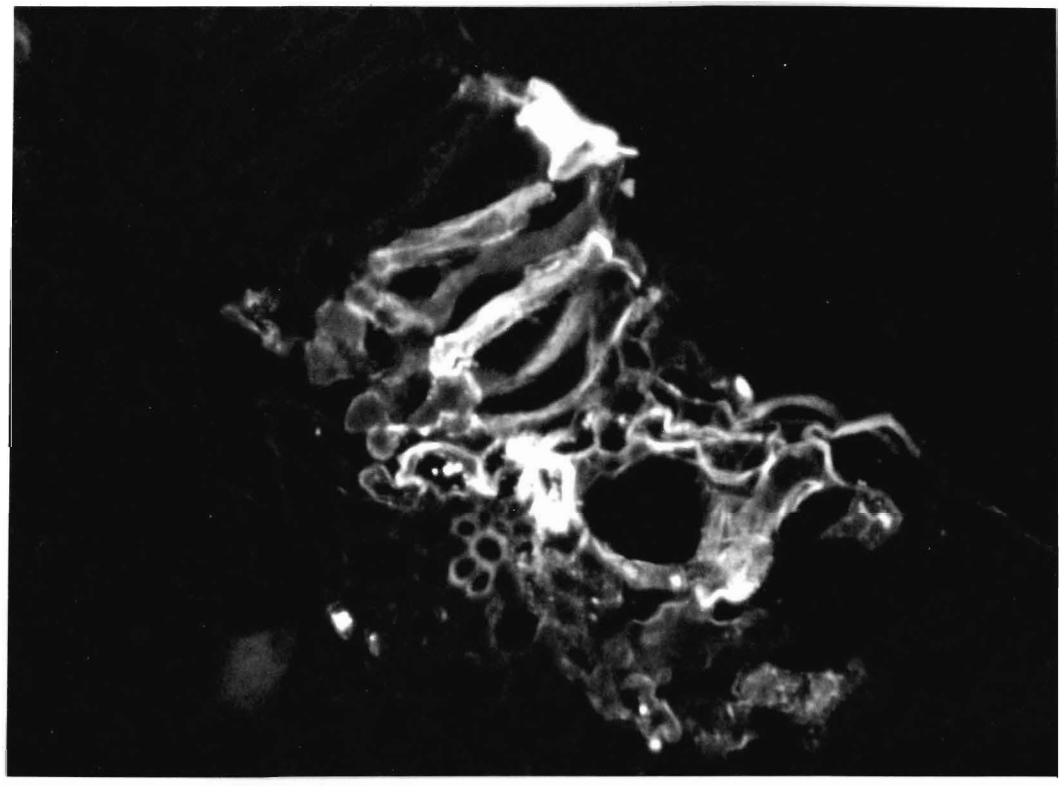
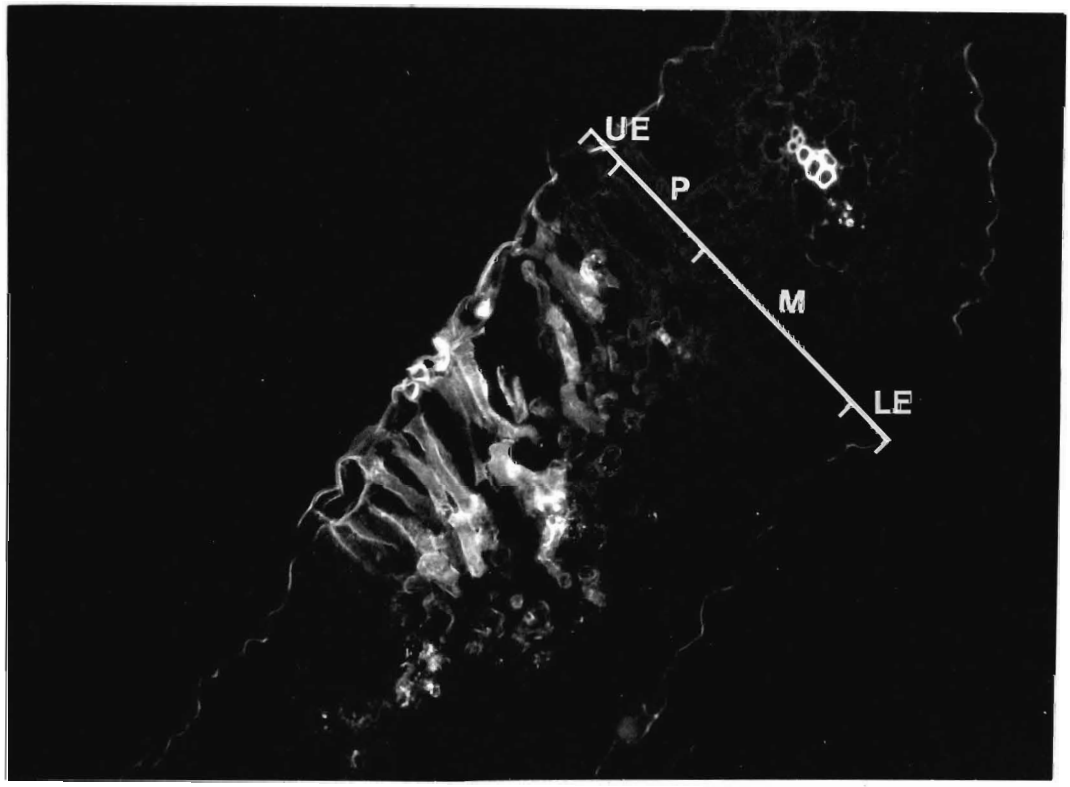


Fig. 24 Fluorescent micrograph of a cross section of a TMV-Pinto lesion showing callose fluorescence in upper epidermis, palisade, and upper mesophyll cells. Callose is identified by white areas bordering cell walls (X300) Stain:Aniline blue

(UE) upper epidermis
(P) palisade cells
(M) mesophyll cells
(LE) lower epidermis


UE
P
M
LE

undergoing necrosis(Fig. 25,26). Fluorescent micrographs of the lesion surface indicated intensified callose fluorescence at the interface between necrotic and healthy cells.

Callose fluorescence was always observed in epidermal cells surrounding the necrotic lesion core. Walls of palisade and mesophyll cells surrounding the necrotic palisade and mesophyll cells exhibited fluorescence when stained with aniline blue, indicating callose deposition throughout the cell walls of these cells(Fig.27,28). The formation of callose was also noted in leaf hairs on the dorsal leaf surface (Fig.29). This deposition was apparent along the basal septa of each trichome. Septations of the basal cells was more clearly defined in scanning electron micrographs prepared from trichome sections(Fig.30). Attempts to determine the time of callose deposition in injured leaf hairs were unsuccessful.

ii. Effect of Elevated Temperatures on Callose Formation

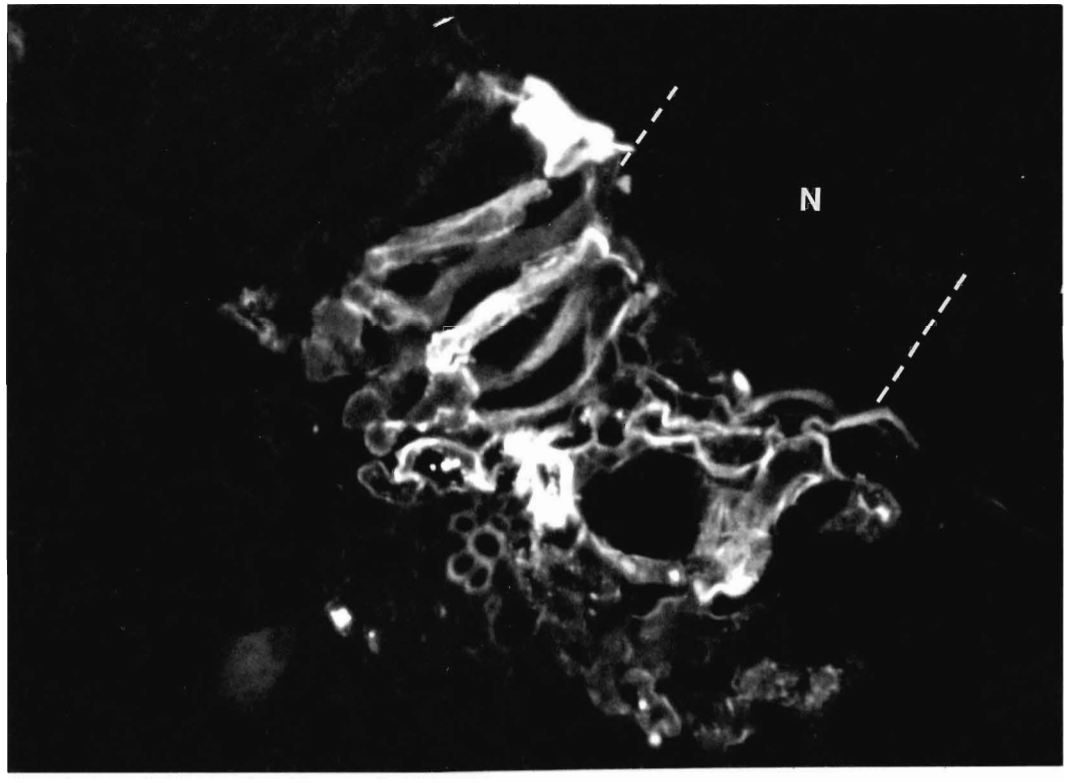
Healthy,celite inoculated, and TMV-celite inoculated bean leaves were incubated at 37°C. under continuous illumination. Leaf tissue was sampled at 12 hr. intervals over a 48 hr. period, and examined for the presence of callose. TMV-celite inoculated leaves incubated at 22°C. were

Fig. 25 Fluorescent micrograph of a cross section through a TMV-Pinto bean lesion showing callose fluorescence (X800) Stain: Aniline blue

(N) necrotic zone

Fig. 26 Cross section of TMV-Pinto lesion (as above) showing corresponding necrotic tissue (X800)

(N) necrotic zone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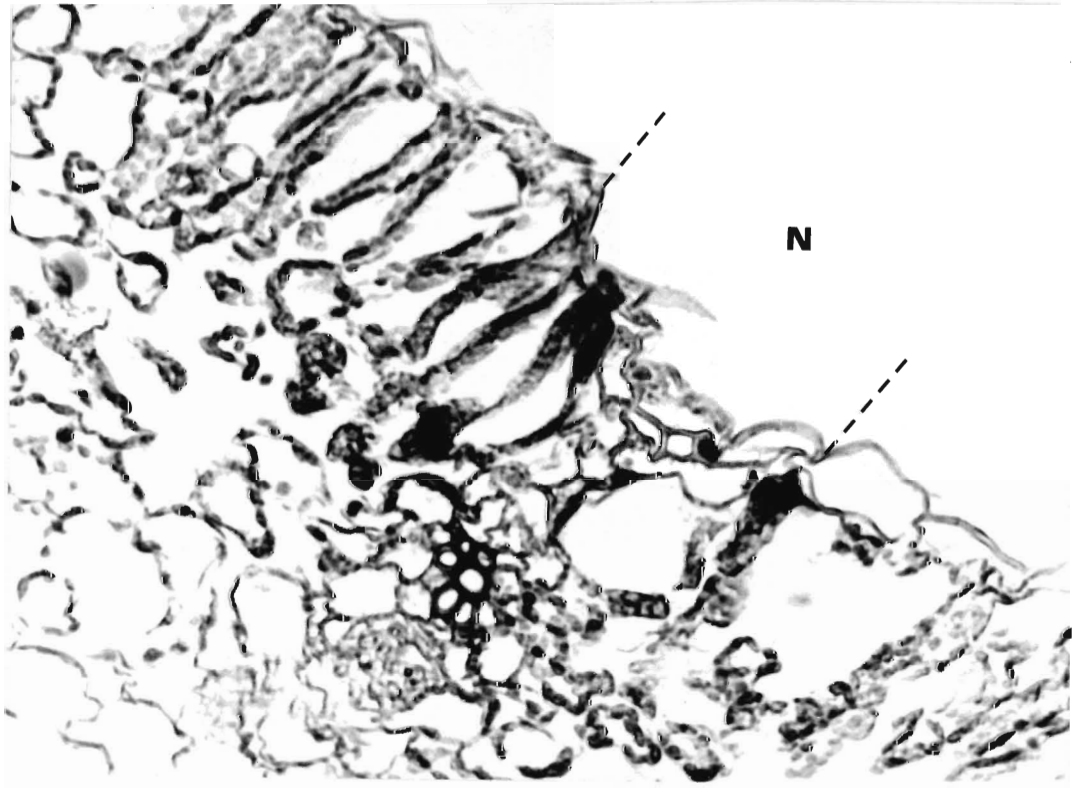
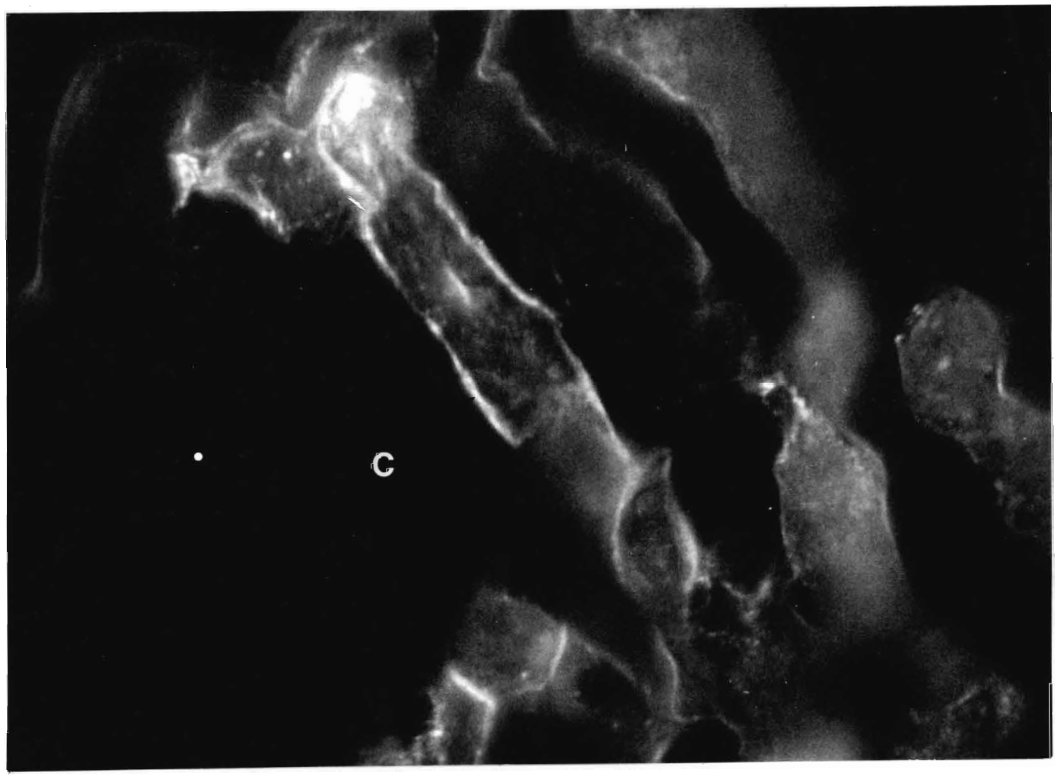

N

Fig. 27 Fluorescent micrograph showing callose deposition(C) along cell walls of palisade cells (X1,800)
Stain: Aniline blue

Fig. 28 Fluorescent micrograph showing callose deposition(C) along cell walls of upper mesophyll cells (X1,800)
Stain: Aniline blue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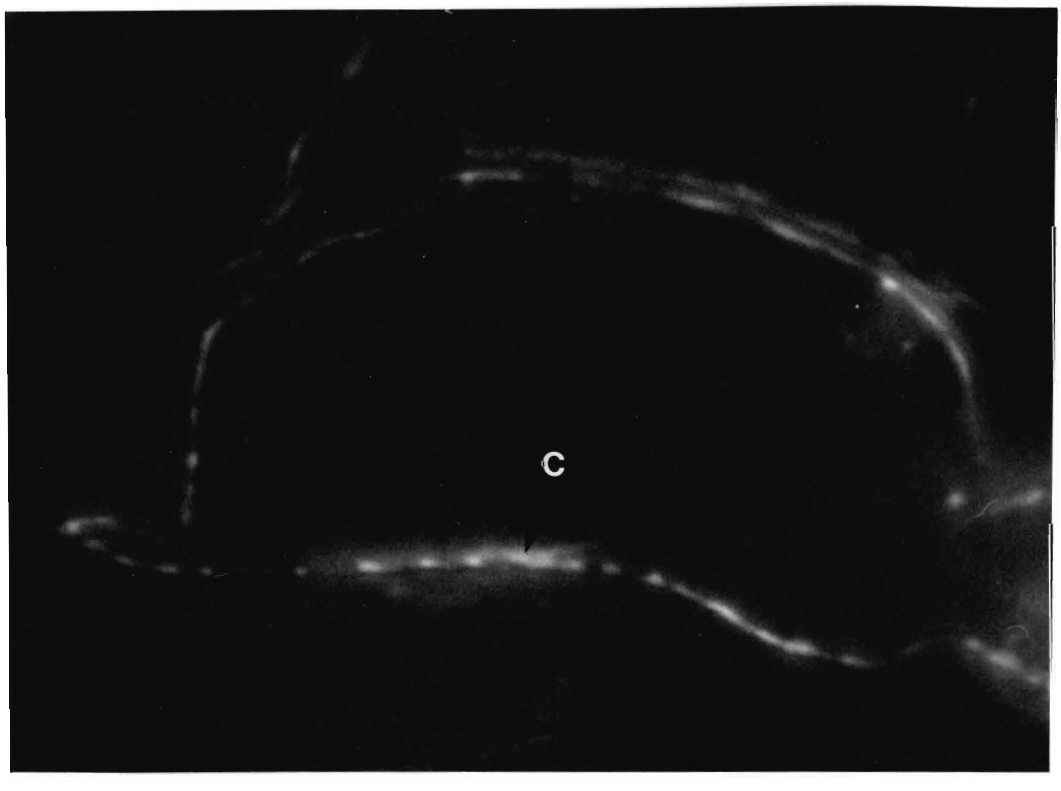
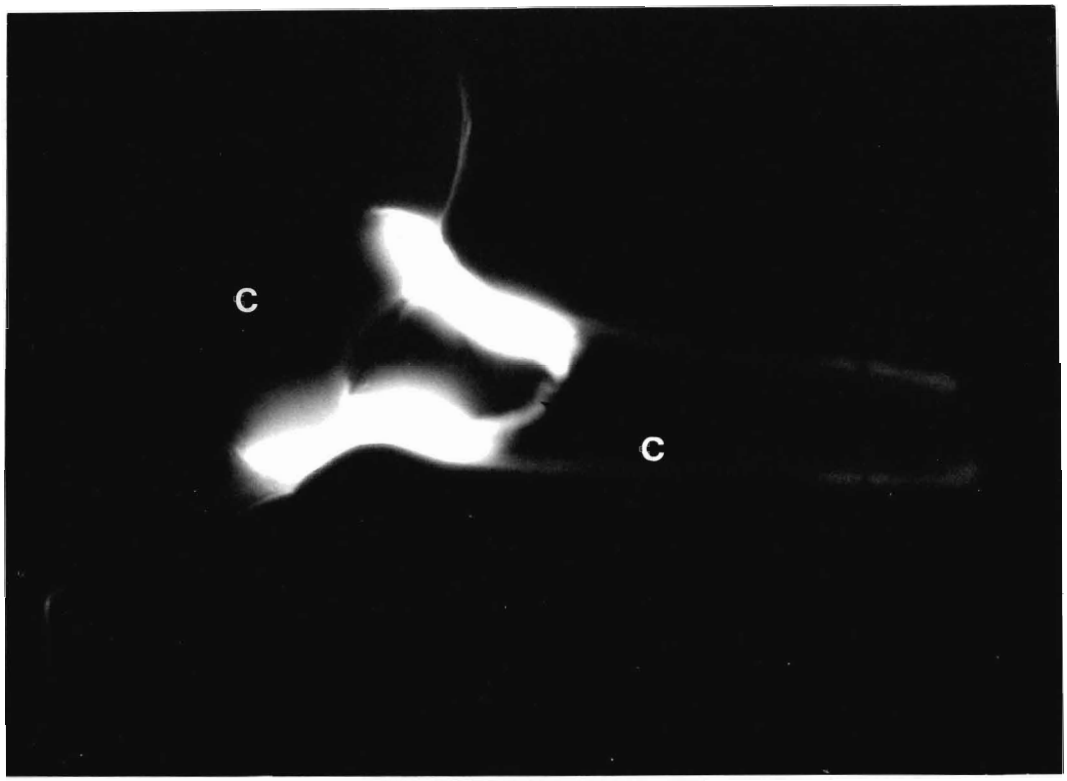
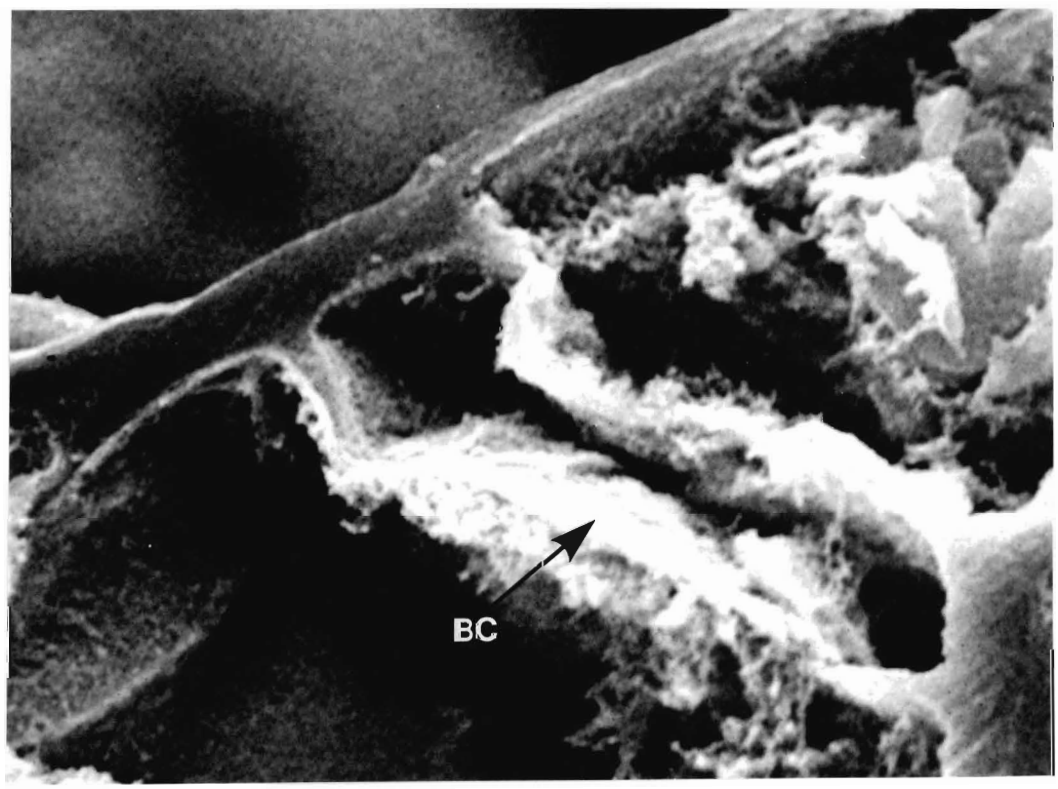

C

Fig. 29 Fluorescent micrograph showing callose deposition(C) along basal cells of a leaf hair sampled from a TMV-Pinto bean lesion. Intense white fluorescence at the base of the leaf hair does not represent callose fluorescence (X1,500) Stain: Aniline blue

Fig. 30 Scanning electron micrograph of a leaf hair basal cell sampled from a TMV-Pinto bean lesion. (X3,400)


C
C


BC

treated the same way as healthy controls.

Leaves incubated at 37 °C. did not show distinct callose fluorescence over the test period(Table 7a). Trace fluorescence resembling that of callose was noted in scattered necrotic cells. Callose was observed at 36 hrs. in TMV-celite inoculated leaves incubated at 22°C., that corresponded with the appearance of lesions.

iii. Effect of Ascorbate Infiltration on Callose Formation

Following vacuum infiltration of primary Pinto bean leaves with 0.05M ascorbic acid, the leaves were briefly rinsed in running water and prepared for inoculation. A second series of controls consisting of water infiltrated leaves were also inoculated. Leaves were floated on their respective infiltration solutions and incubated at 22°C ± 2°C. under continuous illumination(800 ft. candles). Sampling was at 12 hr. intervals over a 48 hr. period. Sections were examined for the presence of callose.

Callose fluorescence was not detected in leaf tissue infiltrated with ascorbate(Table 7b). No necrosis was apparent. Leaf tissue infiltrated with water demonstrated callose fluorescence concurrent with lesion expression at 36 hrs. Intensified callose fluorescence was noted 48 hrs. after inoculation.

Table 7a Time of callose formation in tissue raised at 37°C as determined by light and fluorescent microscopy.

Id: indeterminate
+ : callose detected
- : no callose detected

Sample	Time after inoculation(hrs) 0	12	24	36	48
Healthy	-	-	-	-	-
Celite	-	-	-	-	-
TMV-Celite	-	-	-	-	-
TMV-Celite (22°C)	-	-	-	+	+

Table 7b Time of callose formation in tissue infiltrated with ascorbate as determined by light and fluorescent microscopy.

+ : callose detected
- : no callose detected.

Sample	Time after inoculation(hrs) 0	12	24	36	48
Healthy & AA	-	-	-	-	-
Celite & AA	-	-	-	-	-
TMV-Celite & AA	-	-	-	-	-
TMV-Celite	-	-	-	+	+

iv. Formation of Callose in Injured Tissue

Bean leaf tissue was mechanically injured by applying the blunted end of a needle to the leaf surface. Sufficient pressure was applied to result in necrotisation. Leaves were incubated at 22°C. under continuous illumination (800 ft. candles)over a 36 hr. period. Samples for callose detection were collected at 6 hr. intervals after wounding.

Callose fluorescence was noted as early as 18 hrs. after wounding, however was more predominant at 24 hrs.(Table 8). Callose deposition was confined to cells immediately surrounding the necrotic area.

3. Scanning Electron Microscope Observations

Scanning electron microscope examination of Pinto bean leaf tissue revealed turgid, polymorphic epidermal cells compactly arranged in an irregular lattice(Figs.31-33). At low magnification untreated leaf surfaces were covered by numerous leaf hairs. These hairs consisted of a cylindrical stalk whose diameter decreased towards the apex, which was generally hooked(Figs 31,32). Numerous scattered stomata were clearly seen(Fig.32,33). Traces of foreign material were noted at higher magnification. Mild abrasion of the leaf surface removed the leaf hairs leaving the basal foot cells exposed (Fig.34). Injury to the leaf surface was generally limited to

Table 8 Time of callose formation in injured leaf tissue as determined by light and fluorescent microscopy

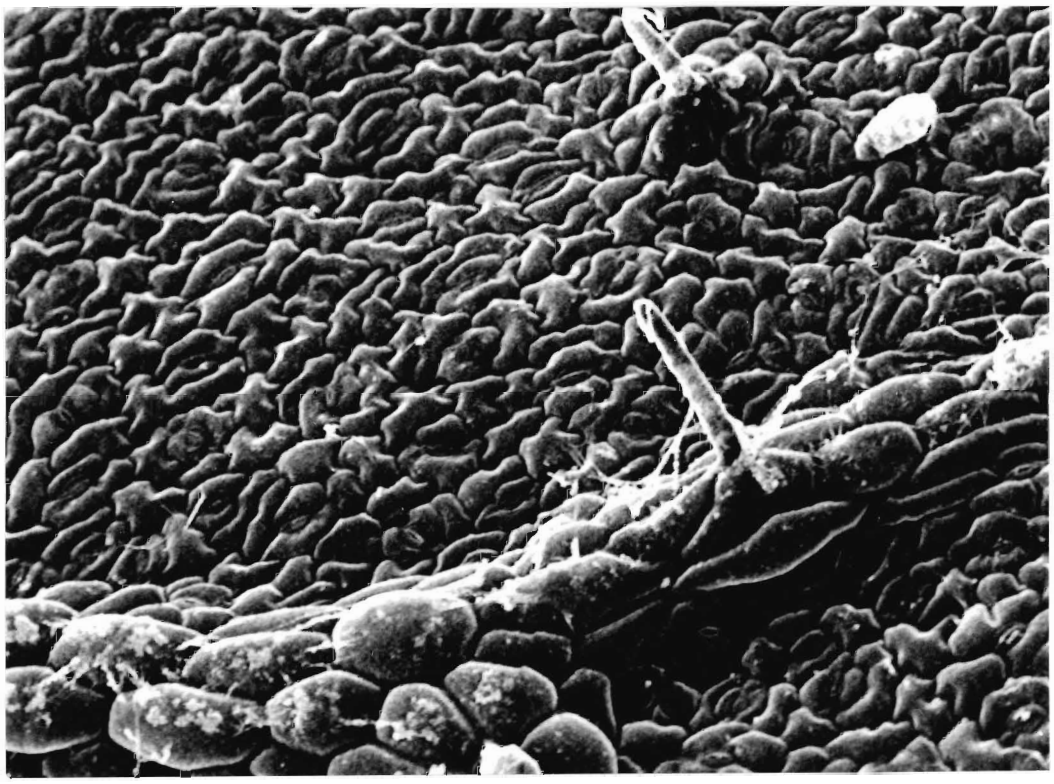
Id: indeterminate
+ : callose detected
- : no callose detected

Time after wounding (hrs)	Callose stains		
	Resorcinol	Lacmoid	Aniline blue
0	-	-	-
6	-	-	-
12	-	-	-
18	-	-	+
24	-	+	+
30	+	+	+
36	+	+	+

Fig. 31 Scanning electron micrograph of the surface of healthy Pinto bean leaf tissue (X200)

Fig. 32 Scanning electron micrograph of the surface of healthy Pinto bean leaf tissue (X400)

(H) leaf hair
(S) stomata



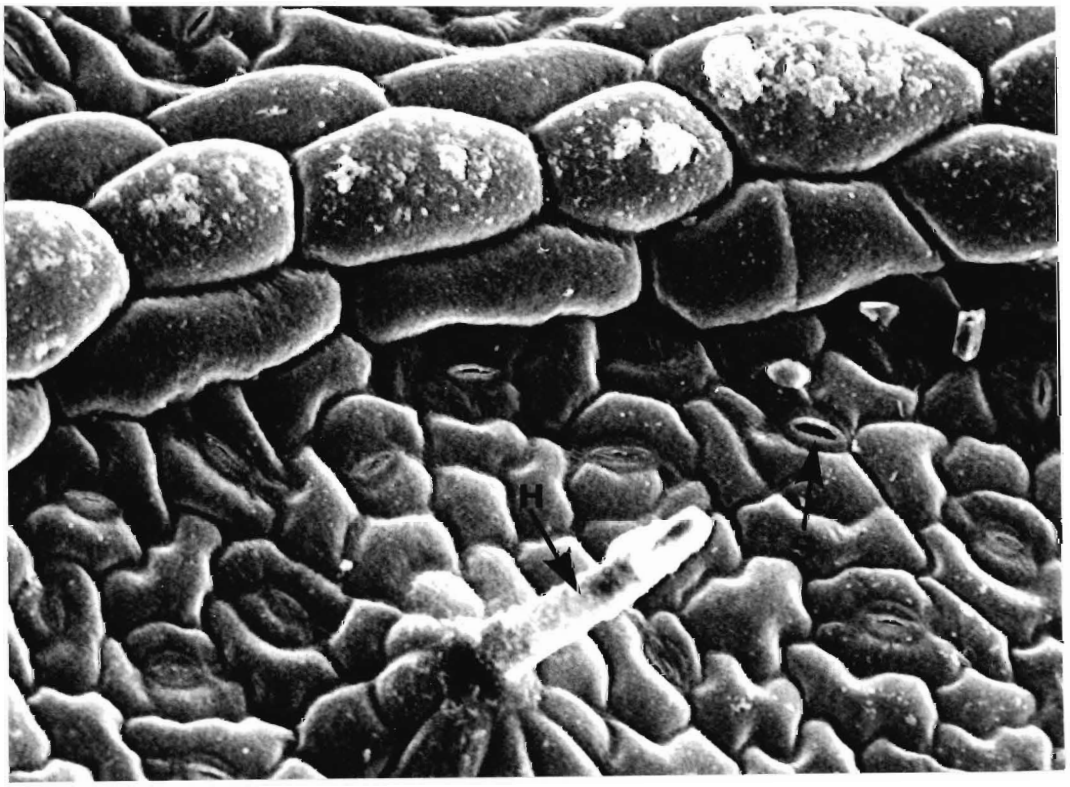
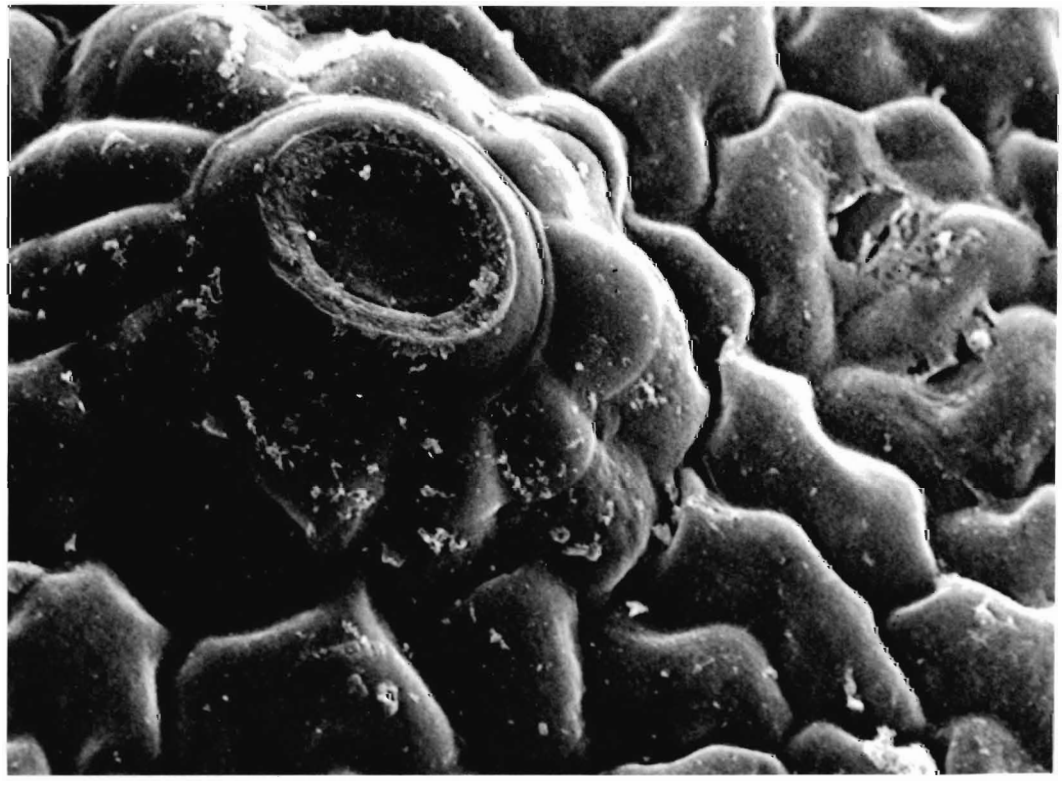

H

Fig. 33 Scanning electron micrograph of epidermal cells in healthy Pinto bean leaf tissue (X2,300)

(s) stomata

Fig. 34 Scanning electron micrograph of a damaged leaf hair resulting from mild abrasion of the leaf surface (X1,200)


S



mild scratching of the cuticular layer of the epidermal cells. In extreme cases, deep wounding caused cuticle displacement or fracturing.

Surface views of necrotic local lesions produced by TMV showed irregular concavities bordered by ridges(Figs.35,36). Collapsed necrotic epidermal cells characterised the lesion tissue(Figs. 37,38) outlined by turgid epidermal cells. Collapsed stomatal guard cells were frequently observed in the lesion tissue(Fig.37).

Cross sections through bean leaf tissue revealed intact epidermal, palisade and mesophyll layers(Figs. 39,40). Most cells, undamaged during sectioning, retained a high degree of turgidity. Elongated palisade cells were typified by smooth walls with few surface abnormalities. Less compact mesophyll tissue consisting of irregularly arranged globular cells, formed a continuum between palisade and lower epidermal cells.

Sections taken through infected tissue disclosed severe necrotic collapse in the upper epidermis and palisade parenchyma (Fig.41). Severe lateral constriction was apparent in palisade cells and cell extremities were irregular and torn (Fig.42)

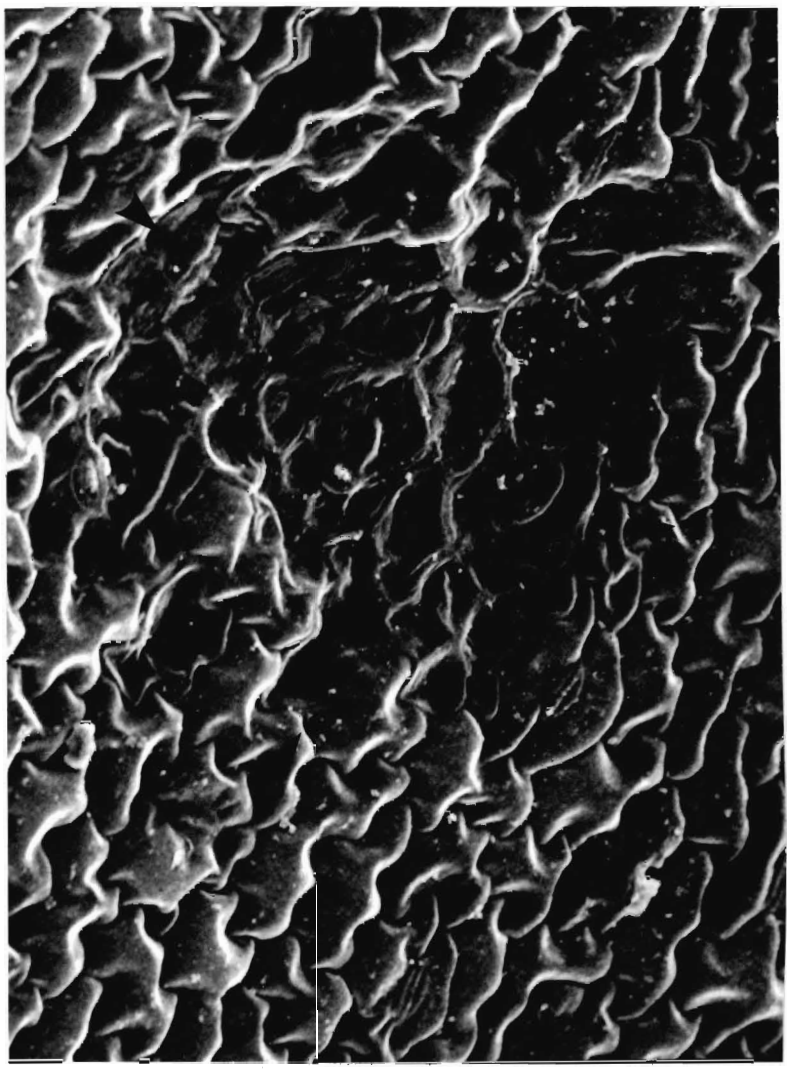
4. Electron Microscope Observations

i.Sites of Virus Synthesis

Previous investigations(Stobbs,1973) indicated that TMV was present in small quantities in the necrotic tissue. Higher virus concentrations occurred at the lesion periphery.

Fig. 35 Scanning electron micrograph of the surface of a lesion in TMV infected Pinto bean (X200)

Fig. 36 Scanning electron micrograph of the surface of a lesion in TMV infected Pinto bean (X400)



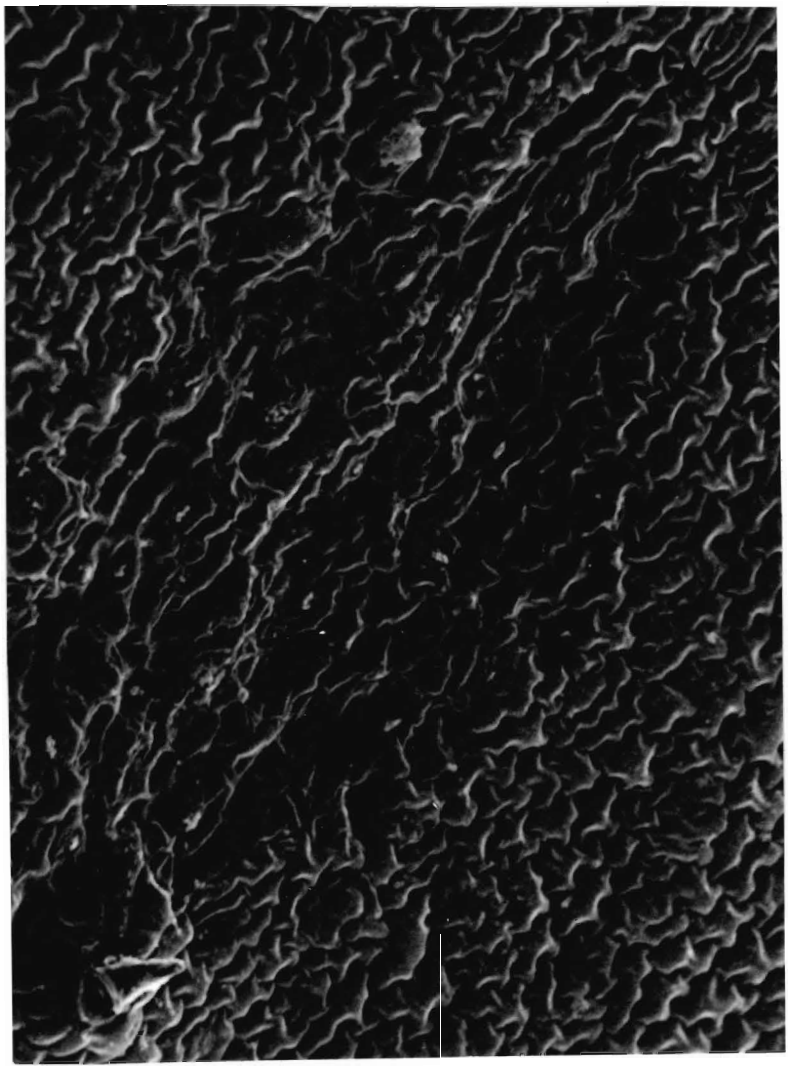
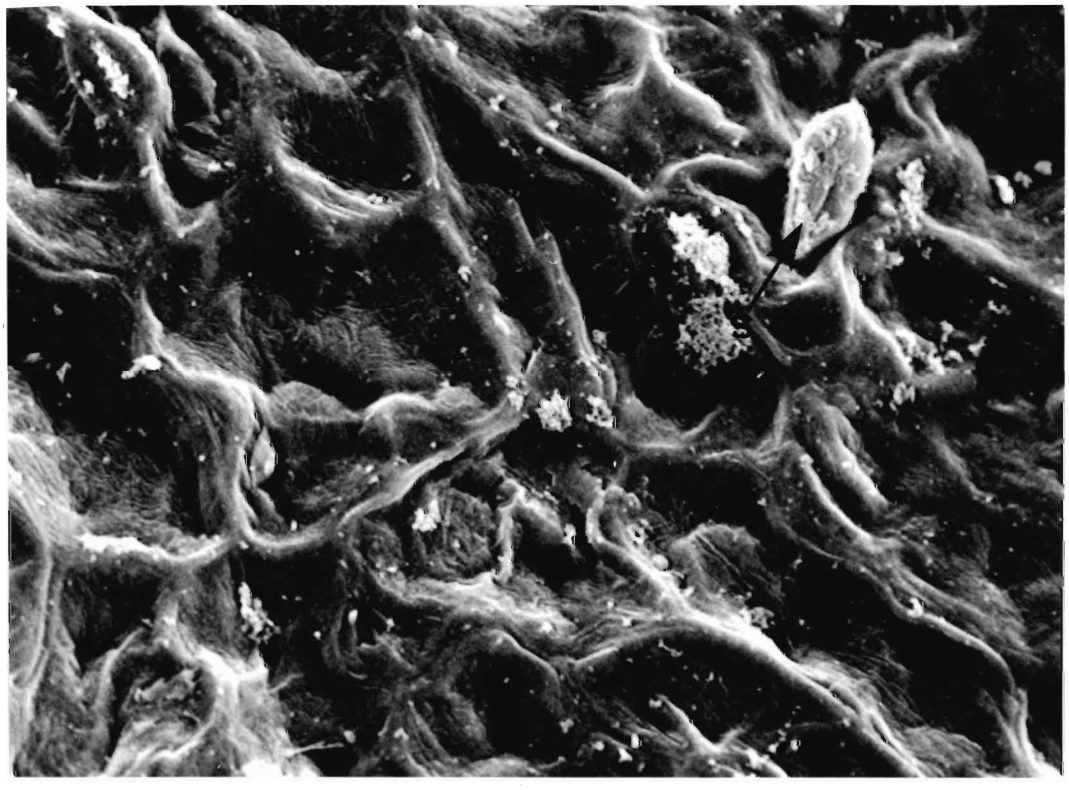


Fig. 37 Scanning electron micrograph of the surface of the lesion centre illustrating necrotic epidermal cells and collapsed stomata(S) (X1,200)

Fig. 38 Scanning electron micrograph of the surface of the lesion centre illustrating cuticular scratches(➤) induced by abrasive action on viral inoculation (X2,300)



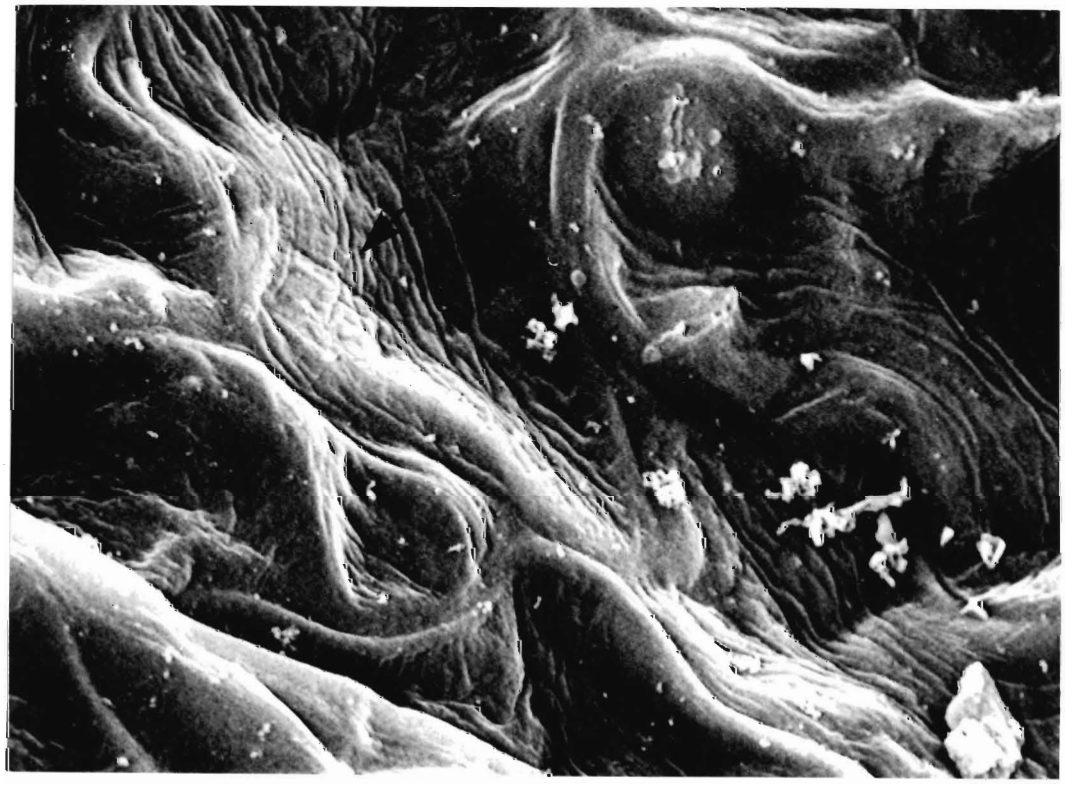


Fig. 39 Scanning electron micrograph of a cross section through healthy Pinto bean leaf tissue (X600)

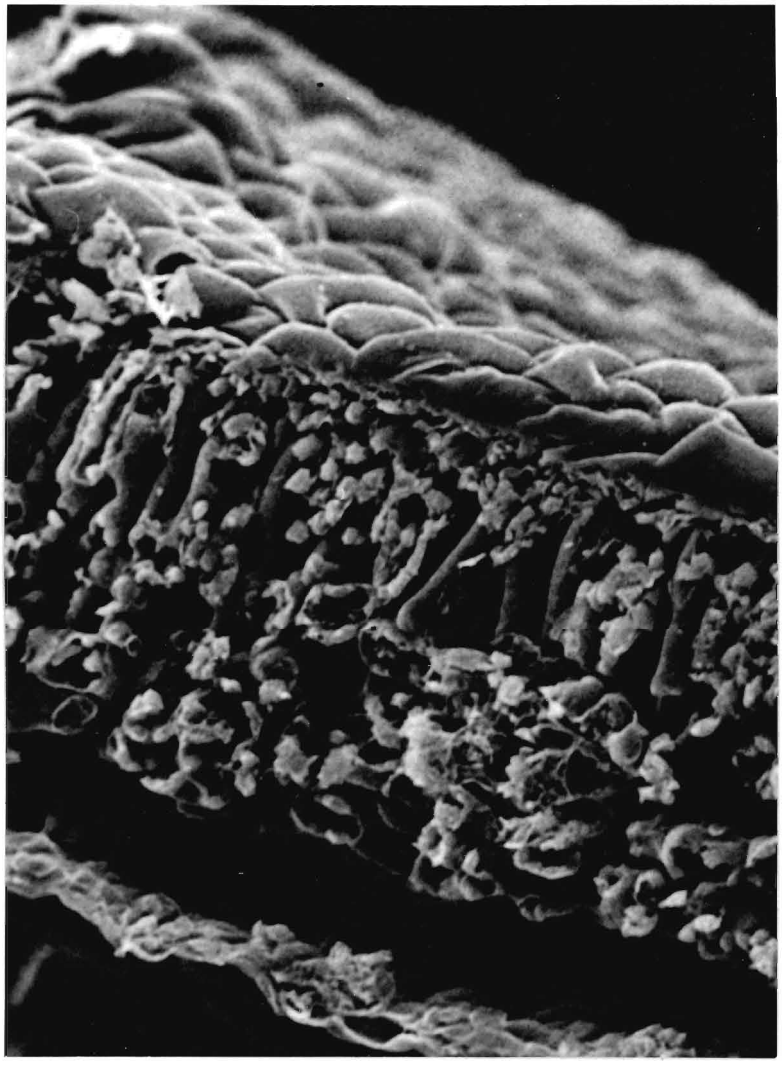
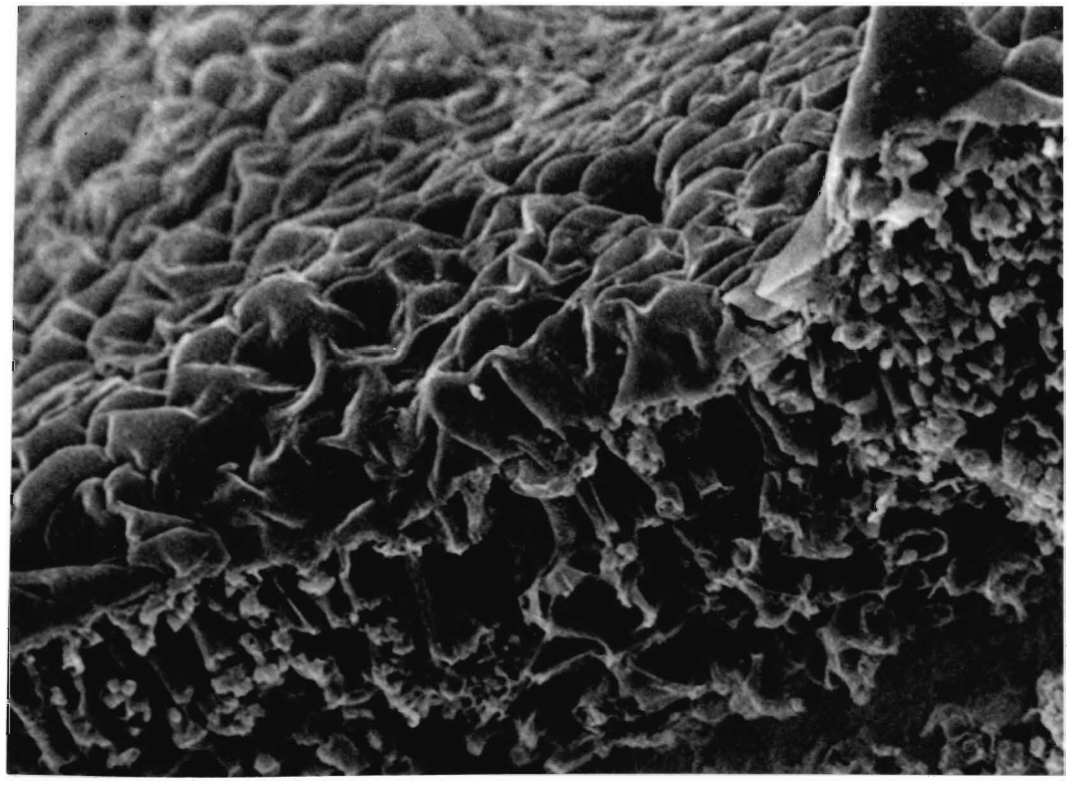


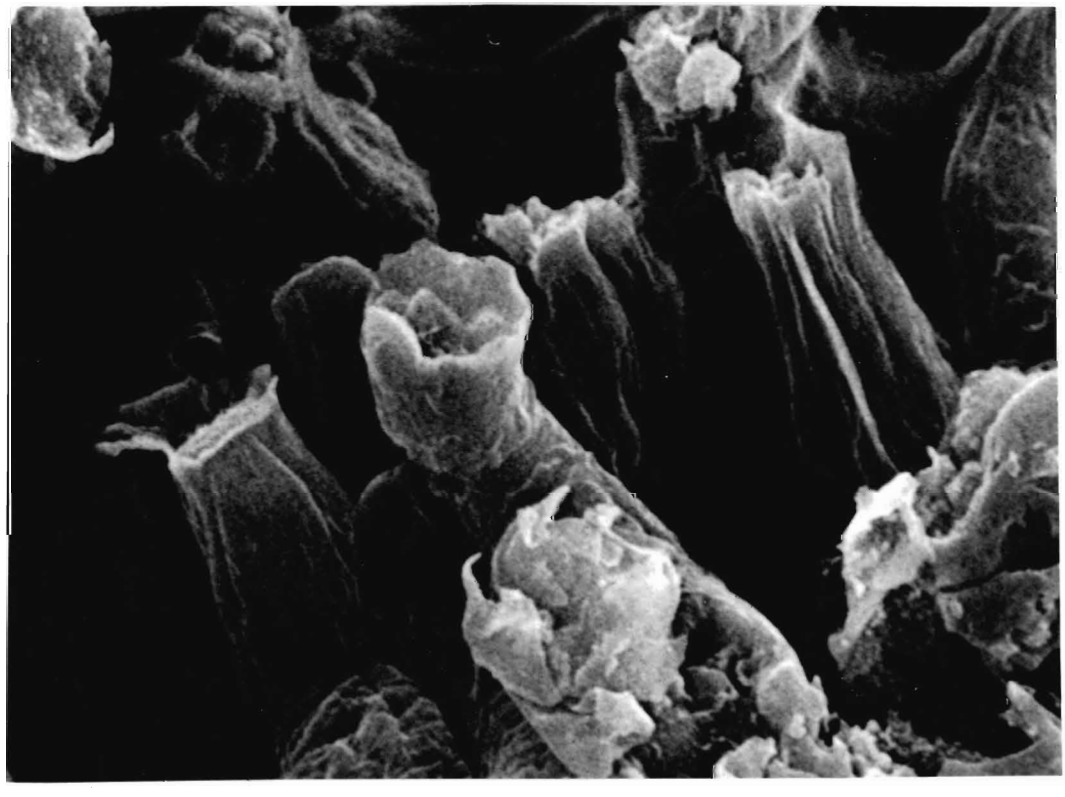
Fig. 40 Scanning electron micrograph of a cross section through healthy Pinto bean leaf tissue illustrating intact epidermal(E), palisade(P), and mesophyll cells(M) (X2000)


E
P
M

Fig. 41 Scanning electron micrograph of a cross section through a lesion in TMV infected Pinto bean leaf tissue (X500)

Fig. 42 Scanning electron micrograph of a cross section of palisade cells in a TMV-Pinto bean leaf lesion, illustrating severe constriction and distention of the palisade cells (X2,800)





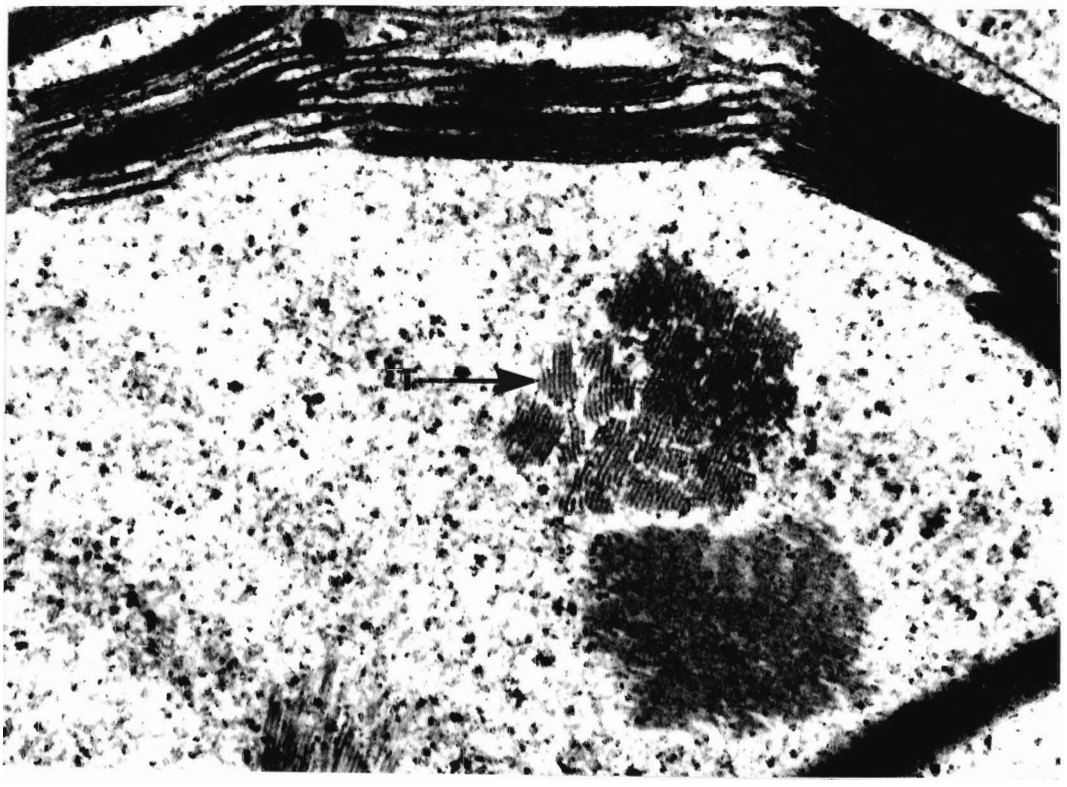
In 56 hr. infections, mono and multilayered crystalline aggregates of rod-like particles were observed in the chloroplasts(Figs.43-44). Numerous rod shaped particles were observed aggregated in the cytoplasm(Fig.45). At high magnification, these particles were discernible as rods, approximately 300 nm in length and 15 nm in diameter(Fig.46). An electron opaque core approximately 5 nm in diameter orientated parallel to the long axis of the particles was seen in several of the rods.

ii. TMV Localisation in Lesion Tissue

Sampling transects(as described under Materials and Methods) are shown in Fig. 47. Large numbers of TMV rods were noted in the marginal tissue bordering the lesion(Fig.48). Virus rods were not detectable in areas outlying the lesion margin. Lesser amounts of virus was noted in the necrotic lesion centre. TMV rods were noted in abundance in the upper epidermis and palisade layers of the lesion tissue. Only trace amounts of virus was found in the mesophyll tissue. TMV was not detected in the lower epidermis. TMV was not observed in the outer areas of fluorescent metabolite deposition (Fig.19).

Fig. 43 Electron micrograph showing presence of TMV inclusions (T) in chloroplast in a TMV infected Pinto bean lesion (X50,000)

Fig. 44 Electron micrograph of TMV inclusions in a chloroplast in a TMV-Pinto bean lesion. (X50,000)



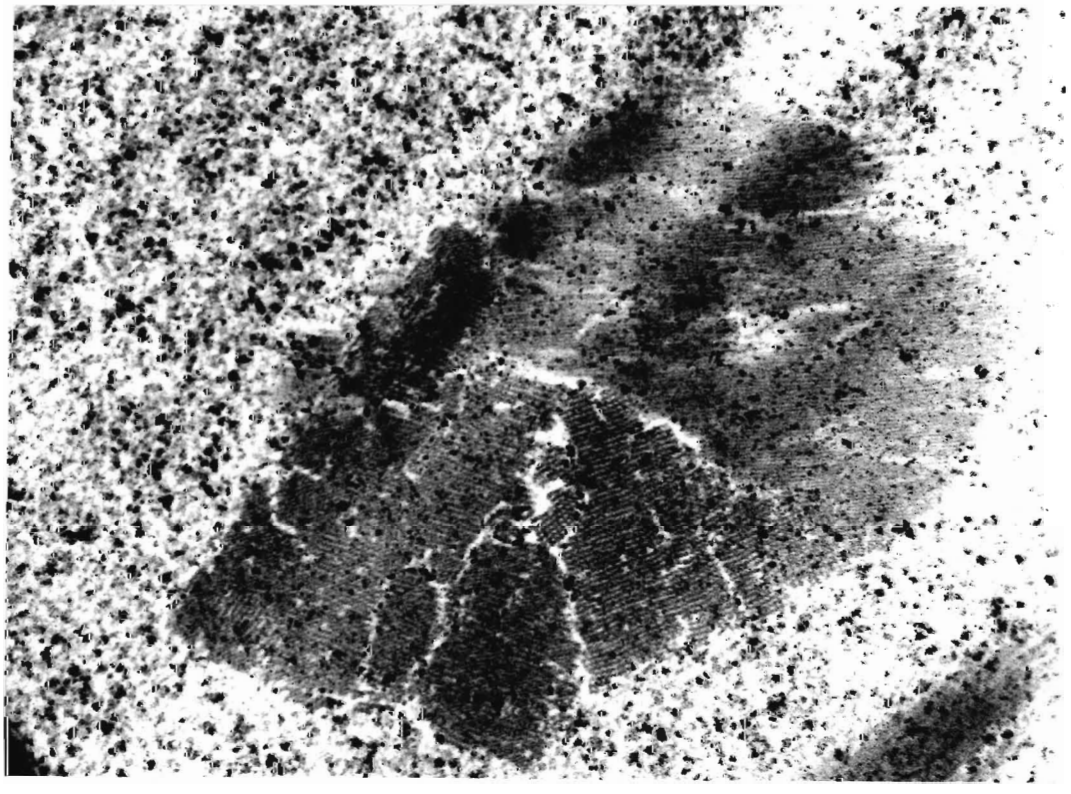
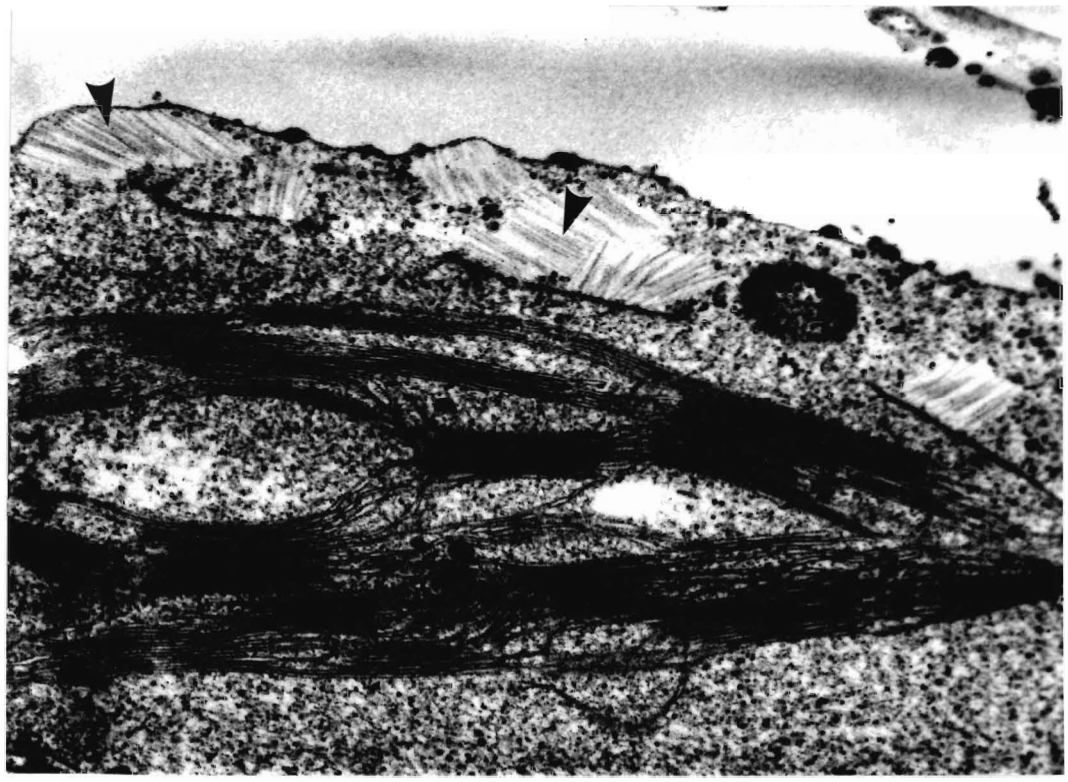
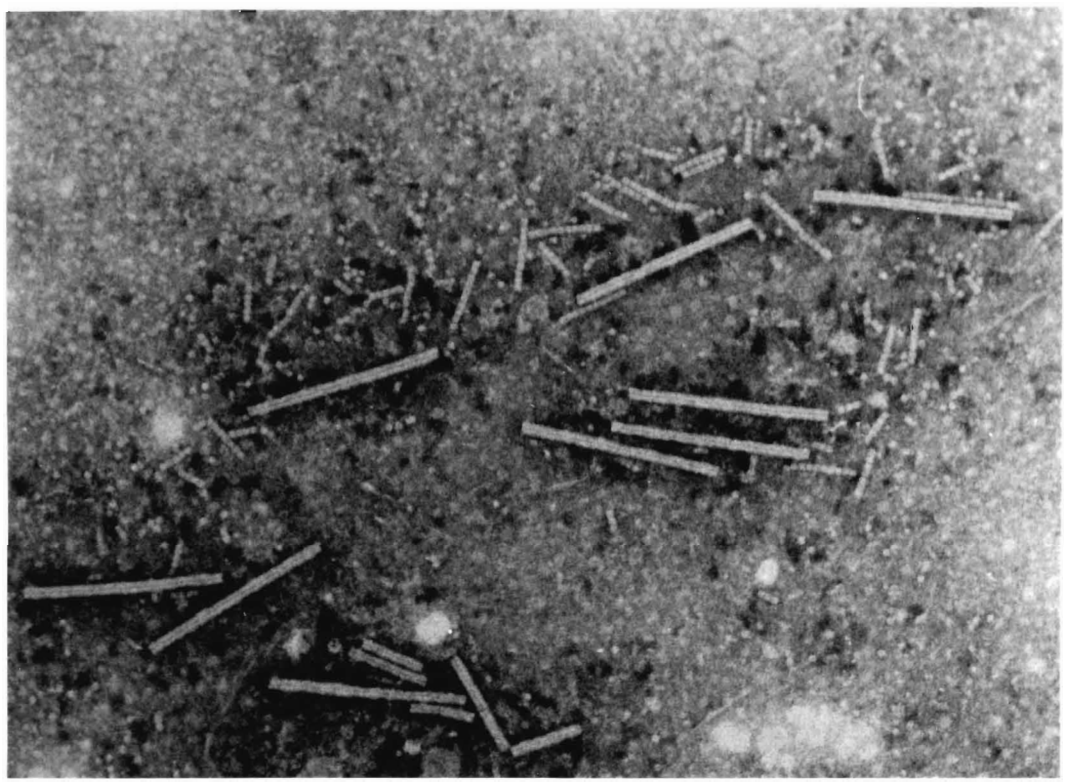


Fig. 45 Electron micrograph of virus particles (➤) in cytoplasm of TMV infected Pinto bean leaf tissue (X30,000)

Fig. 46 Electron micrograph of TMV particles (➤) in a macerated sample of infected Pinto bean leaf tissue (X80,000)





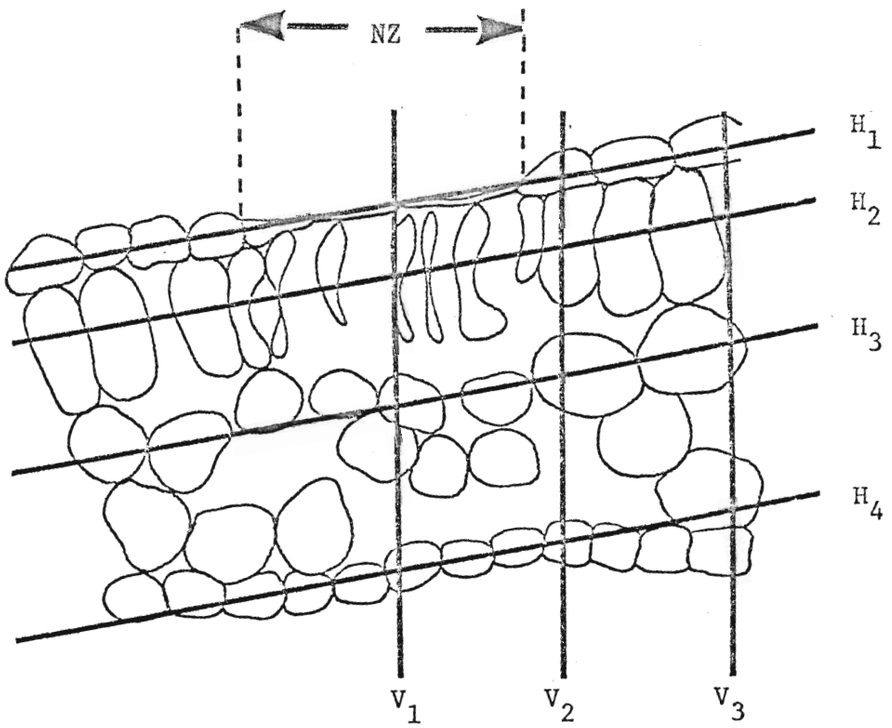


Fig.47 Sampling transect positions examined for virus (V) vertical, (H) horizontal, (NZ) necrotic zone.

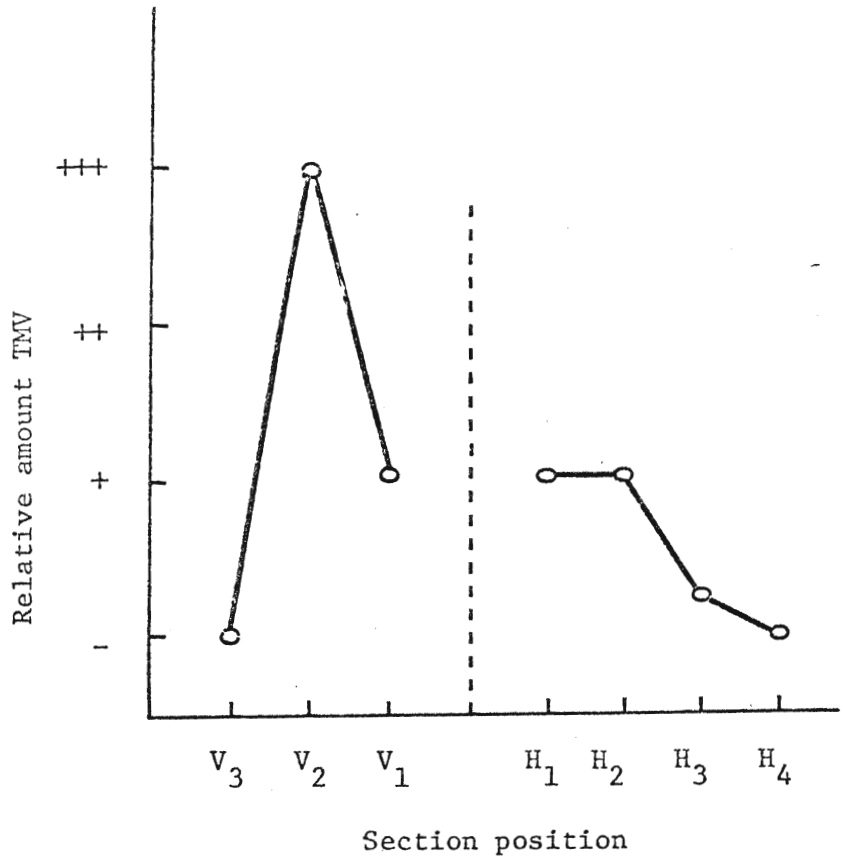


Fig. 48 Relative amounts of virus determined at sampling transect sites (V) vertical, (H) horizontal

DISCUSSION

The hypersensitive reaction of *Phaseolus vulgaris L. var Pinto* to tobacco mosaic virus(TMV) infection is characterised by the production of aberrant phenolic compounds and the appearance of necrosis around the infection sites. It was of interest to investigate these changes induced on virus infection to better understand their association with the restriction of virus spread in infected host tissue. To discuss the mechanism of the hypersensitive reaction, it is useful to examine hypersensitivity at both the biochemical and histological levels.

Changes in the phenolic composition of bean leaves following TMV infection has been examined. The chromatographic behavior and colour reactions of these metabolites with several histochemical reagents permits specification of a number of their structural properties. Among the metabolites common to healthy, mechanically injured, and TMV infected leaves, derivatives of quercetin, caffeic acid, and *p*-coumaric acid were tentatively identified(Fig.2). Three prominent extra-fluorescent metabolites (metabolites 11.12,13,Fig.1) recorded in TMV infected leaf extracts were similar in R_f mobilities and fluorescent properties to those metabolites previously reported in TMV infected Pinto bean leaves(Gill,1965). However unlike the metabolites recorded by Gill, no reactions were apparent with phenol, quinone, amino acid or organic acid reagent indicators. In addition, on acid hydrolysis, the extra-fluorescent metabolites determined in

our work were found to react with diazo salts. The reaction of these metabolites with diazotised sulfanilic acid establishes the presence of an unsubstituted position para or ortho to one of the hydroxy groups of a phenol(Metabolites 17,19,Table 2b). The failure to produce a red colour with Arnow's reagent may indicate the absence of an *o*-dihydroxy structure(Metabolite 19, Table 2b). The relatively low concentration of the extra-fluorescent metabolites may account for the lack of reactivity with certain phenolic spray indicators(Folin Ciocalteau reagent, Hoeptner's reagent). The failure of the extra-fluorescent metabolites in unhydrolised bean leaf extracts to react with a wide spectra of phenolic reagents suggests the *in vivo* complexing of the phenolic moiety of these metabolites in infected tissue. *In vitro* experiments with tobacco streak virus(TSV) infected tobacco leaves have demonstrated that the quinone of chlorogenic acid binds to protein to form a complex which yields caffeic acid on hydrolysis(Hampton,1970). Enzymatically generated quinones have been shown to react with terminal nitrogen of primary amino groups, aliphatic amino groups, and thiol groups in amino acids(Mason and Peterson,1965.,Spurr and Main,1974). Oxidation of tannins to quinones with subsequent polymerisation is believed to result in the production of various phlobaphenes (Barker and Sasser,1959.,Christie,1949.,Dropkin and Nelson,1960). In our experiments, attempts to isolate and characterise the various acid hydrolysates of the extra-fluorescent metabolites were unsuccessful.

Further separation of the unhydrolised extra-fluorescent metabolites revealed six distinct metabolites(Fig.4). These closely resembled those reported by Gill(1965) in tobacco necrosis virus(TNV) infected Pinto bean leaves. Similarity of these metabolites produced by either spherical(TNV) or rod(TMV) virus infections suggests a production in the host of certain metabolites specific to virus infection. In fact, these metabolites were not found in bean leaves infected with bean rust(*Uromyces phaseoli*) or common blight(*Xanthomonas phaseoli*). However, the review of the literature showed that too few pathogens have been studied to conclude that such a correlation is in fact valid or is a simple coincidence.

Two extra-fluorescent metabolites(metabolites 11,12, Table 4b) were detected in extracts from severely abraded leaves and TMV-celite inoculated leaves. The co-appearance of these metabolites in both mechanically injured and infected leaf tissue indicates that such substances may be regarded as host metabolites induced in injured cells. The present work shows that these metabolites were associated with the appearance of the necrotic symptoms and they increased with the severity of cellular necrosis(Table 4a,b). Both wounded and infected tissues exhibited similar blue-green fluorescent bands extending 4 to 5 mm. beyond the necrotic margins. The localised accumulations of these compounds is in some respects similar to the accumulations of scopoletin in tomato spotted wilt virus and TMV lesions in

Nicotiana tabacum and *Nicotiana glutinosa* as reported by Best(1944,1963). Such fluorescence has been attributed to phenols of the phenylpropanoid type(Frigit et al,1973).

To better ascertain the significance of such extra-fluorescent metabolites, it is necessary to understand their relationship to the basic necrotic process. Attempts to study the role of the extra-fluorescent metabolites in the hypersensitive reaction have been limited by the absence of techniques for the accurate localisation of these metabolites within the infected tissue. Fluorescent microscope observations of TMV lesions in Pinto leaves revealed the presence of fluorescent halo rings beyond the lesion perimeter(Fig.19). Such fluorescence, limited to cells undergoing necrosis and those cells immediately adjacent to them, is likely associated with the impending necrobiotic process. Various biochemical and physiological changes in halo tissue in various host-parasite interactions have been attributed to the diffusion of toxic metabolites from the infection site(Farkas et al,1960). If such metabolites are causally associated with cellular necrosis, their presence in cells in advance of infection would result in a manifestation of events contingent with necrosis. The necrosis of such cells would be instrumental to the viral localisation of the hypersensitive reaction. Leaves bearing newly formed lesions when subjected to extreme temperatures were found to undergo rapid enlargement within 12 hrs.(Fig.9). Such increased lesion expansion was noted to occur within the fluorescent halo

area. These cells, if already undergoing changes induced by abnormal metabolite diffusion from adjacent necrotic cells, would be highly sensitive to temperature injury and pathogen infection and would explain the rapid lesion expansion(Fig.9). As the lesion enlarges, changes induced in the adjacent cells by the metabolites would render these cells less labile to virus movement to a point where virus multiplication is stopped because the cells are dead.

Our work showed that the incubation of TMV inoculated leaves at elevated temperatures or infiltration of the leaves with ascorbate resulted in an increase in lesion size(Figs 9, 11). Leaves incubated at elevated temperatures or infiltrated with ascorbate did not exhibit necrotic symptoms and extra-fluorescent metabolites were not found(Fig. 5a,b). However on returning the leaves to a lower temperature or on the removal of the ascorbate, lesions appeared and became larger than those of untreated infected tissue. This does not resolve the necrosis-virus limitation contraversy. If necrosis is responsible for limiting virus spread, one would expect virus to spread further in leaves treated either with elevated temperatures or ascorbate infiltration. Preliminary investigations suggest that increases in lesion size may be correlated with increased virus spread(Farkas et al,1960; Stobbs, 1972). Further attempts to study virus movement on necrotic inhibition by ascorbate infiltration (cf. Fig.47,48) were unsuccessful, owing to technical difficulties inherent in the sampling procedure.

Bawden(1964) notes however that *Nicotiana glutinosa* leaves with fully necrotic lesions developed systemic symptoms when exposed to high temperature. If viral localisation was a consequence of necrosis, virus should have been confined by the necrotic cells and lesion size should have remained static. In our experiments, application of a similar procedure to well-developed TMV lesions on Pinto bean leaves failed to produce a similar response, although lesions were of slightly larger diameter(Appendix 4). Similar results were also obtained when ascorbate treatment was discontinued following infiltration for several days(Appendix 5). So it would appear that additional factors other than necrosis may be involved in the mechanism of virus localisation.

If it was possible to determine the exact moment when virus localisation takes place within the cell, its relationship with the necrotic process could be better understood. Such a determination in time is one of the major obstacles in understanding hypersensitive localisation. Much research has been concentrated on interpreting physiological and metabolic alterations occurring at the time of lesion expression that has been believed to be the time of viral localisation. However various contradictions are apparent in the literature based on such interpretations. Early lesion expression is not necessarily coincident with virus localising events. Milo and Santilli(1967) in noting that maximum ascorbate oxidation occurred long after lesion appearance in TMV infected Pinto bean leaves, concluded

that such oxidation had no relationship to the size and development of the necrotic lesion. Our work indicated that lesion expansion in TMV infected Pinto bean leaves is not concluded until some 24 hrs. after initial lesion appearance. Phenolic oxidation products accumulating during this interval may be responsible for incipient necrosis in advance of virus infection. Such oxidation products would produce a rapid decline in endogenous ascorbate levels, correspondent with those reported by Milo and Santilli(1967). Similarily, Tanguy et al(1972) in examining TMV inoculated *Nicotiana tabacum var Xanthi* leaves stated that production and accumulation of phenolic compounds was detectable only several hours after the appearance of local lesions. The authors found that differences in the phenol metabolism were more pronounced 60 to 156 hrs. after inoculation, after which they gradually disappeared. They conclude that since the accumulation and production of phenolic compounds became important only when virus synthesis is completed and hypersensitivity established, the formation of toxic quinones and their polymerised products are unlikely to be responsible for lesion development. Solymosy et al(1967) studying the infection of *Nicotiana tabacum var Xanthi* by TMV conclude that lesion expansion is not concluded until approximately 150 hrs. after inoculation which may indicate that localisation of virus would not be completed until this time. Since the period of pronounced phenol metabolism reported by Tanguy and Martin is found to coincide with the time of lesion

expansion, and since no reference is made to the time of cessation of lesion expansion, the conclusions drawn by Tanguy and Martin(1972) should be subject to reconsideration. Similarily Cabanne et al(1971) in measuring polyphenol-oxidase and peroxidase activity in TMV infected *Nicotiana tabacum L. cv. Xanthi* determined that significant levels of enzyme activity were only apparent after necrosis appeared. They infer that the importance of oxidases in necrotisation is less than has been suggested, and assert that changes in enzyme activity found during the hypersensitive reaction are a consequence, not a cause, of the death of the cells. Since lesion expansion is not likely concluded during the period of time in which their experiment was carried out, the validity of the conclusions drawn by these authors should be subject to similar reconsiderations.

In our work, we found a reduction of circumferal fluorescent deposits following the cessation of lesion expansion in TMV infected Pinto bean leaves. Such a reduction in metabolite(s) production following the termination of necrotic expansion suggests that cellular collapse prevented virus movement to adjacent cells. So cellular necrosis and fluorescent metabolite accumulation ceased.

In the hypersensitive reaction, the oxidation of poly-phenols into toxic quinones takes place(Farkas et al,1960) and ascorbic acid and other reducing agents present in the

cell are diminished. Based on these concepts, we attempted to study the effect of infiltration of ascorbate in bean leaves at varying times following TMV inoculation. Our results showed that a delay in lesion appearance occurred only when leaves were infiltrated prior to a given point in the infection process(Fig.5). Since ascorbate infiltrations were applied for short periods at established intervals during the infection process, they would allow for a temporary reduction of the existing polyphenolic oxidation products at the given time intervals and would delay the induction of necrotic lesion formation. However, if ascorbate infiltrations are carried out after initiation of the necrotic process, no delay in the appearance of lesions will occur. So the time of initial necrotic induction could be determined. Our results showed that the application of ascorbate after 12 to 18 hrs. (Experiment 1 and 2) after TMV inoculation failed to produce a difference in the time of lesion expression between ascorbate and water infiltrated leaves. Such would indicate that the irreversible induction of necrosis occurred prior to this time and well in advance of lesion appearance. Light microscope observations at this time failed to distinquish any changes in overall cell structure. Although no changes were noted, our results showed that additional metabolites not present in the controls could be observed by fluorescent microscopy. Similar metabolites were not detectable however by paper chromatography probably because they were at this time only

present in trace amounts. We believe however that they represent the phenolic compounds associated with the initiation of the necrotic process which later produce the quinones responsible for the hypersensitive reaction.

Further evidence of the initiation of the necrotic events prior to lesion expression is provided by callose studies. Our results indicate that callose formation in mechanically injured tissue was not initiated until 18 hrs. after injury (Table 8). In TMV infected Pinto bean leaves, callose deposition was always observed concurrent with lesion expression. It is therefore apparent that the time of callose initiation coincides with the time established for the necrotic induction of the lesion. Prevention of necrotic induction by either ascorbate infiltration or elevated temperature treatments of infected leaves, similarily was found to prevent callose formation(Table 7a,b).

In our experiments, observation of TMV infected Pinto bean leaves indicated the occurance of callose deposition in semi to non-necrotic cells bordering the lesion. The presence of additional fluorescent metabolites however was noted in advance of this callose deposition. Such metabolites are regarded to diffuse outwards from the necrosing cells contained within the lesion centre. We believe that these metabolites are probably responsible for the stimulation of callose formation in the non-necrotic cells bordering the lesion. Accordingly, such callose deposition would limit further outward

cell to cell movement of the virus resulting in the eventual restriction of lesion size. A suggestion that cell to cell movement of the virus through plasmodesmata(DeZoetin and Gaard,1969., Esau,1967., Kitajima and Lauritis,1969)may be prevented by callose deposition has been recently supported by additional evidence from a TMV-Pinto bean system(Wu et al, 1969,1970). The failure of Spencer and Kimmins(1971) to detect callose in bean tissue infected with TMV may well have resulted from the different areas examined or the method of sampling, as well as the use of different technical procedures.

Callose fluorescence in infected leaf tissue was respondent with similar fluorescence in mechanically injured tissue. Similarities between symptoms induced by viruses and those induced by mechanical injury have been reviewed by Esau(1948). Isolated occurrences of callose fluorescence on celite inoculation are indicative of the small degree of damage sustained by the leaf surface. Although the means by which virus particles penetrate the epidermal cells is not clearly understood, it is clear that mechanical inoculation using abrasives wounds the cuticle and epidermal cells. It is generally assumed that wounding breaks the cell wall and thus allows the virus to enter the inner cytoplasm(Kimmins and Casselman,1969). Virus may then move into adjacent uninjured tissue, presumeably through plasmodesmata. In our work, scanning electron micrographs showed numerous broken leaf hairs in the abraded tissue.

Basal septal cells were delineated by faint callose fluorescence. The degree of callose deposition was correlated with the degree of necrosis produced either by mechanical injury or by the formation of virus necrotic lesions. It is suggested that the effects of TMV infection on Pinto bean foliar tissue are those of an extension of the basic wounding process, such that normal wound healing events are involved in the characteristic expression of virus symptoms. A further inference suggested by these results is that localisation may occur only when transmission of the virus has also caused a wound response to the host. Tobacco mosaic virus *per se* is incapable of inducing local lesions in unwounded tissue(Kimmins and Casselman,1969). Thus the colligative effect of incipient wounding followed immediately by viral inoculation(an effect attained by celite addition to the inoculum) is found to result in rapid callose formation concurrent with tissue necrosis in that region.

It is apparent from the highly destructive changes occuring during the development of a local lesion, that the total organisation of the cells is thrown into chaos. Systems that normally regulate physiological and metabolic activity are disrupted and destroyed, including membrane systems, mitochondria, and chloroplasts. The terminal stages of infection were marked by the increased opacity of the cell. Such darkening presumeably is the effect of polyphenol oxidation and deposition of polyphenols and tannins. The tearing away of

cell walls from adjoining cells may be brought about by changes in the composition of the middle lamella. TMV inclusions, noted in various ordered and dispersed patterns, ranged from irregularly aligned groups of particles to well ordered crystalloids(Figs. 43-45). At high magnification, the electron opaque RNA core was seen in several of the rods after negative staining(Fig.46). The particles observed had the following features in common with purified tobacco mosaic virus examined by Carroll and Shalla(1965): *1*.a rod shape about 300mu in length and 16mu in diameter *2*.a central core of low electron density *3*. a tendency to aggregate in mono or multilayered crystals contained within the cytoplasm. Although one cannot measure the infectivity, the form and intracellular organisation of these structures leaves little doubt as to their being TMV. Failure to demonstrate TMV in various local lesion hosts(Weintraub and Ragetli,1964.,Shalla, 1959) may have been due to preparative procedures.

SUMMARY

1. The phenolic composition of *Phaseolus vulgaris L. var Pinto* was examined following TMV infection. Six extra-fluorescent metabolites, associated with necrotic symptom expression, were isolated and characterized by two dimensional descending paper chromatography. Two of these metabolites also present on mechanical abrasion are considered to represent host metabolites produced in response to injury.

2. The incubation of TMV inoculated leaves either at elevated temperature or in ascorbate solutions was found to prevent lesion expression and extra-fluorescent metabolite formation. Extra-fluorescent metabolite formation has been shown to be coincident with lesion expression. Removal of either the elevated temperature or ascorbate treatment resulted in the formation of enlarged lesions. Such is suggestive of continued viral movement in the absence of necrosis.

3. Ascorbate studies indicated that necrotic events were determined early in the infection process, coincident with trace fluorescent metabolite accumulation in the infected tissue.

4. Callose deposition was observed concurrent with lesion expression. The degree of callose deposition was correlated with the degree of necrosis produced either by mechanical injury or

virus induced necrotic lesions. The time of callose induction was found to coincide with the time established for the necrotic induction of the lesion. Callose deposition was prevented by elevated temperature or ascorbate treatments. The formation of callose in response to necrotisation in the early stages of infection is suggested.

5. Fluorescent microscope studies indicated abnormal fluorescent metabolite deposition in non-necrotic cells outlying the lesion. Callose deposition was confined within this region. The association of these metabolites in the induction of callose formation is suggested.

6. Histological examination of infected tissue revealed extensive upper epidermal and palisade parenchyma damage in the lesion tissue. Mechanical damage to the host during inoculation was minimal. Optimum virus levels were found in the upper epidermal and palisade cells bordering the necrotic lesion. TMV was not detected in areas outlying these cells.

On the basis of the findings of this study, it is apparent that changes occur in the healthy cell as a result of viral infection. Abnormal metabolite production in *Phaseolus vulgaris L. var Pinto* following tobacco mosaic virus infection was strongly correlated with the appearance of necrotic symptoms. Removal of these metabolites and subsequent necrosis resulted

in continued lesion expansion. The causality of aberrant metabolite production with either the initiation of necrosis or the limitation of virus spread however was not possible to confirm using available techniques. Irreversible cellular damage is believed to be initiated early in the infection process, coincident with the stimulation of callose forming events. Changes in cells in advance of infection are believed responsible for the limitation of lesion expansion and restriction of virus spread.

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APPENDIX 1

SOURCE OF SEEDS

SEED	SUPPLIER
Phaseolus vulgaris L. var Pinto	Haney Seed Co., P.O. Box 502, Twin Falls, Idaho, USA.
Nicotiana tabacum cv. 'harrow velvet'	Canada Dept. of Agriculture Research Station, Vineland Station, Ontario.

APPENDIX II

NUTRIENT SOLUTION (James,1963)

Constituents	Wt./litre water.
$CaSO_4 \cdot 2H_2O$	0.25g.
$CaH_4(PO4)_2 \cdot 2H_2O$	0.25g.
$MgSO_4 \cdot 7H_2O$	0.25g.
NaCl	0.08g.
KNO_3	0.70g.
$FeCl_3 \cdot 6H_2O$	0.005g.

Dissolve in one litre of water. $FeCl_3 \cdot 6H_2O$ is added prior to use.

APPENDIX III

INDICATOR REAGENT PREPARATION

Aluminum chloride reagent(Geissman,1955) 1% alcoholic solution of aluminum chloride. For the detection of flavonoids.

Ammonium molybdate reagent(Lederer and Lederer,1957) 2.5% w/v on 0.2N HCl. Purple-blue colouration with Xanthate.

Arnow's reagent(Arnow,1937) 10g. sodium nitrite and 10g. of sodium molybdate dissolved in 100 ml. water. Detection of *o*-dihydroxy phenolic compounds.

2,4 dinitrophenylhydrazine(Reio,1958) 500 mg. 2,4-dinitrophenylhydrazine dissolved in 1000 ml. hot 1N HCl. Solution filtered before use. Detection of aldehyde and keto groups.

Ehrlich's reagent(Pridham,1959) 1% solution of *p*-dimethylaminobenzaldehyde in 1N HCl. Store under refrigeration. Detection of amino acids and peptides.

Ferric chloride reagent(Reio,1958) 2% $FeCl_3 \cdot 6H_2O$ in water used. General reagent for phenolic compounds, unspecific.

Folin-Ciocalteu reagent(Bray and Thorpe,1954) diluted 1:1 with distilled water. Detection of phenols and other easily oxidizable substances.

Hoeptner's reagent(Roberts and Wood,1951) 5% w/v $NaNO_2$ in 0.9N acetic acid followed by 1N NaOH. Determination of dihydric phenols.

Lead acetate reagent(Pridham,1959) 1% aqueous basic lead acetate. Detection of flavonoid compounds.

Ninhydrin reagent(Block,Durrum, and Zweig,1955) 0.3% ninhydrin in 95% ethanol followed by heating at 100'C for 10 min. Detection of amino acids and biogenic amines.

Diazotized nitroaniline(Bray and Thorpe,1954) 25ml. of a solution of *p*-nitroaniline(0.3%) in HCl(80% w/v) added to 1.5 ml. of $NaNO_2$ solution(5%w/v) prior to spraying. Subsequently chromatogram is sprayed with Na_2CO_3(20%w/v in water). Detection of hydroxybenzoic acids and aminohydroxybenzoic acids.

Phosphomolybdate reagent(Reio,1958) 2% aq. solution of $H_3PMo_{12}O_{40} \cdot 29H_2O$ used. Overspray with ammonia. Detection of phenolic compounds and their esters.

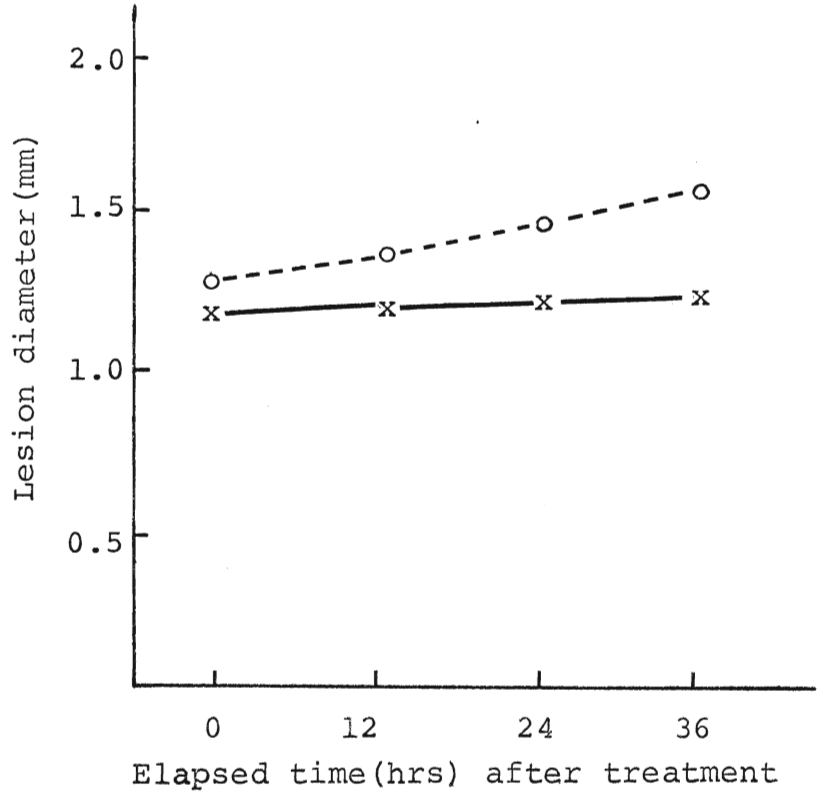
Potassium permanganate reagent(Reio,1958) 1% aq. solution of $KMnO_4$. Detection of carboxyl groups.

Diazotized sulfanilic acid(Linskens,1959) 25 ml. of freshly prepared 5% $NaNO_2$ is slowly added at 0'C to 5 ml. sulfanilic acid solution(0.9g. sulfanilic acid and 9 ml. of concentrated HCl, dilted to 100 ml. with water). Dried chromatograms oversprayed with 20% Na_2CO_3. Establishes the presence of an unsubstituted position para or ortho to one of the hydroxyl groups.

Sodium molybdate(Pridham,1959) 0.1M aq. solution of sodium molybdate. Detection of *o*-dihydroxyphenols.

APPENDIX IV

Lesion diameter change following heat treatment of fully expanded lesions(96hr.) for 36 hrs. Lesion diameter recorded over a 36 hr. period following the conclusion of heat treatment.

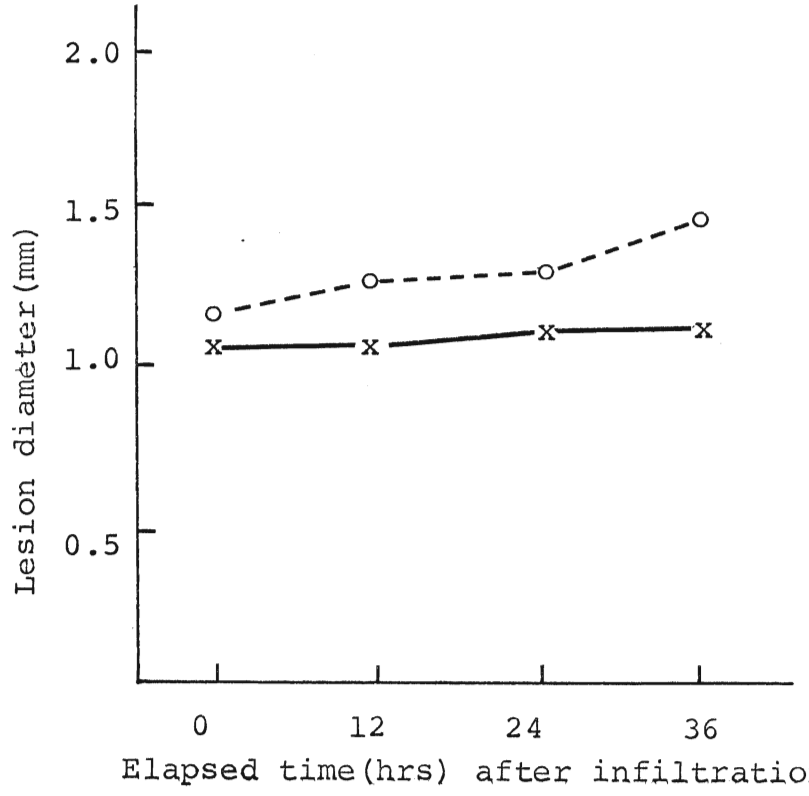


0----0 Leaves heat treated at 37°C.

x——x Leaves heat treated at 22°C.

APPENDIX V

Lesion diameter change following the infiltration of ascorbic acid(0.05M) into fully expanded lesions(96hr.) Ascorbic acid treatment was maintained for 36 hrs. On discontinuation of treatment, lesion diameter was recorded over a 36 hr. period.



o----o Leaves infiltrated with ascorbic acid

x----x Leaves infiltrated with water

All incubations at 22°C.